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THE RELATION BETWEEN BLOOD DESTRUCTION AND THE OUTPUT OF BILE PIGMENT.

By G. O. BROUN, M.D., PHILIP D. McMASTER, M.D., AND PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 11, 1923.)

Many facts point to a close relation between blood destruction and the yield of pigment in the bile. The assumptions currently made in this connection by physiologists and clinicians have recently been summarized by one of us and critically reviewed.[1] A method now available for total bile collection[2] renders possible an experimental test of some of them.

The Pigment Changes Following Bile Loss.

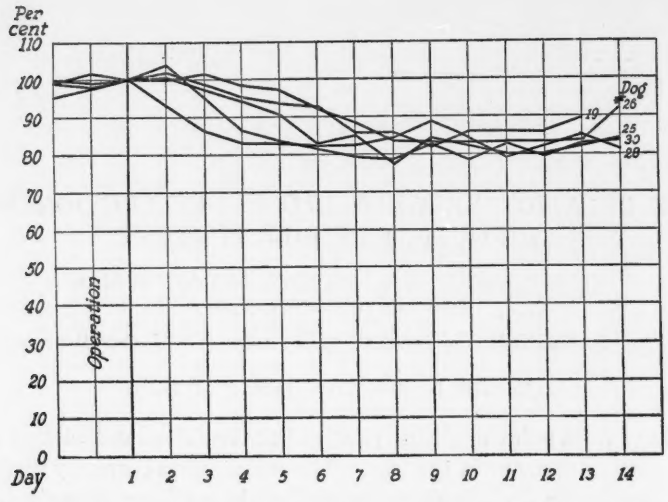
In dogs losing all their bile, significant changes take place in the hemoglobin percentage of the blood and in the bilirubin content of the secretion lost. They have been briefly described in a previous paper in which the methods employed for the care of the animals and the study of the bile have also been outlined.[3] As a rule, for several days after the operation whereby drainage of the common duct is effected, the hemoglobin percentage alters but little (Text-figs. 1 and 2), though in general it tends to fall slowly to about four-fifths of the initial value in the course of a week (Text-fig. 1). The bilirubin output by contrast falls off briskly from the beginning, and at the end of the same period of time averages only about half of the first amount (Text-fig. 3).[4] There can be no doubt that the large early amount is sometimes derived in part from extravasated blood. The yield from dogs in which hemorrhage into the tissues was known to

[1] Rous, P., *Physiol. Rev.*, 1923, iii, 75.

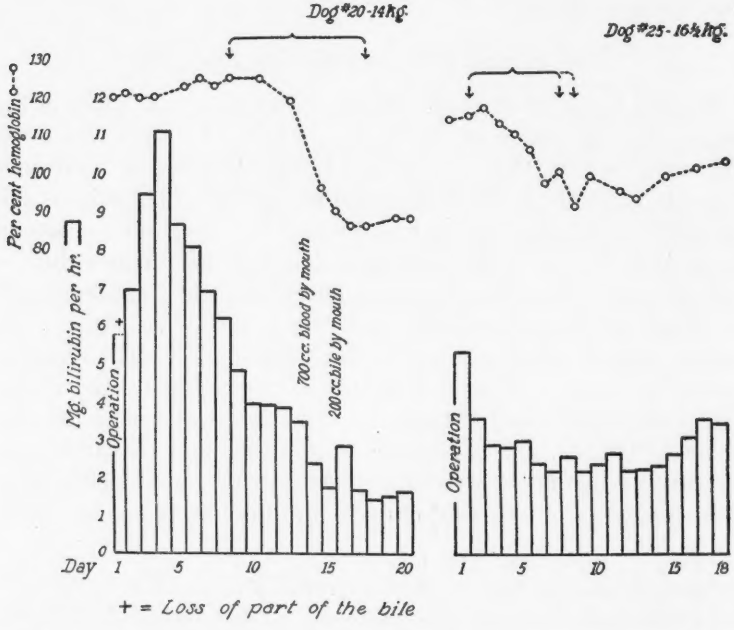
[2] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.

[3] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

[4] See also McMaster, Broun, and Rous,[3] Text-fig. 2. Several instances complicated by blood extravasation are there included.

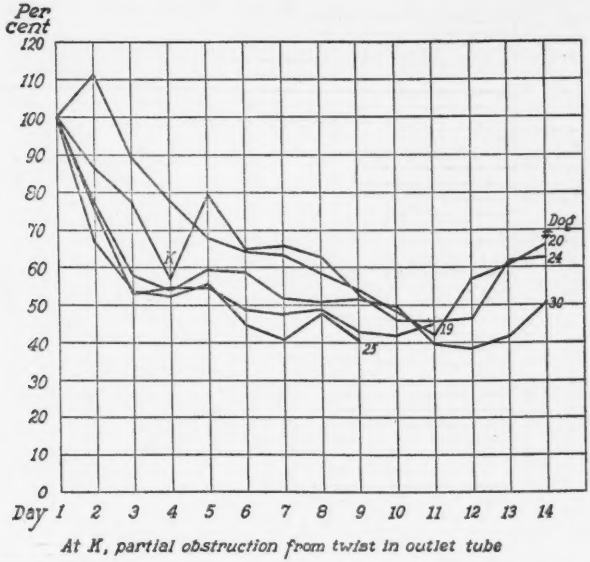


TEXT-FIG. 1. Changes in the hemoglobin percentage expressed in terms of the finding on the 1st day after operation.—Uncomplicated instances.



TEXT-FIG. 2. (See Table I.) Unusually marked postoperative changes in the hemoglobin percentage and bilirubin output. The periods dealt with in Table I, during which the hemoglobin percentage fell rapidly, are included between the bracketed arrows on the chart.

have taken place increased markedly during the period when the hematoma was breaking down (as, for example, in Dog 30 of Text-fig. 6). Yet when careful hemostasis was practised, and at most only a few cubic centimeters of blood left the vessels, the pigment yield halved in from 7 to 10 days (Text-fig. 3).

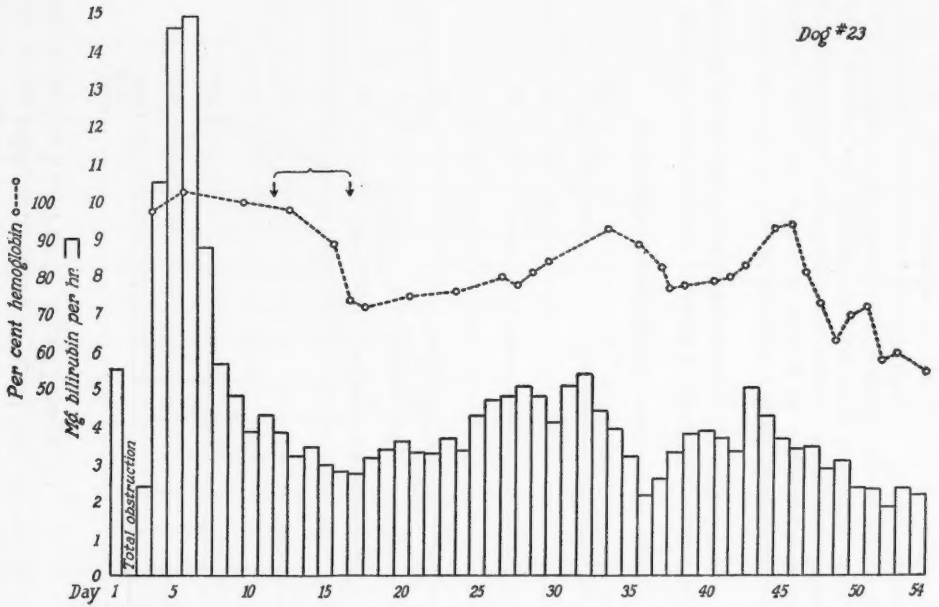


TEXT-FIG. 3. Postoperative changes in the bilirubin output in uncomplicated instances, as expressed in percentages of the yield of the 1st day.

There has been no general recognition of these postoperative changes for the excellent reason that animals in which a bile fistula has been established after the classical fashion are ordinarily not worked with until wound healing has taken place. Brugsch and Retzlaff[5] alone appear to have noted them, remarking as an aside to other matters that during the first 14 days of bile loss there is a falling off in number of red cells and bilirubin output. They furnish no figures on the point. Their dogs were suspended in slings and all the bile was collected.

The later pigment changes vary with the individual. In our animals they were unquestionably affected by certain of the experi-

[5] Brugsch, T., and Retzlaff, K., *Z. exp. Path. u. Therap.*, 1912, xi, 508.



TEXT-FIG. 4. (See Table I.) Concurrent changes in the hemoglobin percentage and the bilirubin output during the course of a marked progressive anemia. The periods dealt with in Table I, during which the hemoglobin percentage fell rapidly, are included between the bracketed arrows on the chart.

mental procedures employed, notably by exercise[3] and by bile feedings.[6] But always in uncomplicated instances, the anemia indicated by the early drop in the hemoglobin percentage persisted, though varied, at times, by intervals of more or less complete recovery.[7] Usually it remained slight. We are inclined to believe that in the two animals in which it became marked (see Text-fig. 4; also McMaster, Broun, and Rous[8]), there was another factor involved besides loss of the bile. Dog 23 of Text-fig. 4 of the present work had a severe intercurrent bronchitis, and later a gradually developing intestinal obstruction; while Dog 24 of the previous paper[8] occasionally lost blood from hemorrhoids. With mild anemia the hemoglobin percentage as a rule altered but slightly from week to week, as did the bilirubin output also.[7] Every well defined blood change found reflection in a change in this last. In the two dogs just mentioned, with pronounced anemia, remissions occurred at frequent intervals, the hemoglobin percentage rising for a time only to fall again, usually to a lower level; and as it rose and fell so too did the yield of bilirubin. The subjective element can be ruled from account in connection with the findings; for the estimations on blood and bile were made by different workers each unaware of the other's results, while, furthermore, the remarkable correspondence in the pigment changes did not come to attention until the data were correlated, weeks after they had been obtained. The synchronous alterations were slow and wave-like, often as much as a fortnight elapsing between crest and crest. Circumstances have prevented us from following them for more than 3 months.

The character of the anemia was repeatedly studied. Always it was of secondary type, as shown by cell counts and the examination of stained smears. The color index was somewhat, but not greatly, less than the normal; poikilocytosis, though present, was never marked. The number of circulating reticulated cells, which was followed by Robertson's[9] technique, proved to be even less than in healthy dogs.[10]

[6] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 699.
[7] McMaster, Broun, and Rous,[3] Text-fig. 5.
[8] McMaster, Broun, and Rous,[3] Text-fig. 4.
[9] Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.
[10] Krumbhaar, E. B., *J. Lab. and Clin. Med.*, 1922, viii, 11.

The inference seems justified that the hematopoietic tissue was less active than usual. Certainly it was putting forth cells somewhat deficient in pigment.

The charts leave no room for doubt that a quantitative relationship of some sort exists between the blood and bile pigments. But is it the relationship generally supposed? The calculations in vogue nowadays on blood destruction as expressed in the yield of bilirubin or urobilin have for basis the assumptions that from the hemoglobin molecule 4 per cent by weight of hematin is derived, and that for every gram of hematin approximately 1 gm. of bilirubin is excreted.[11] Do the data of our experiments accord with such a view?

Stadelmann[12] long ago noted that after injections of hemoglobin the expected rise in the bilirubin content of the bile was incompletely realized. His finding seems to have been wholly lost sight of. Recently Whipple has sponsored the conception that bilirubin has other sources besides the blood,[13] a principal one being the carbohydrates of the food. We have corroborated the observations of Whipple and Hooper[14] on the effects of carbohydrate diet to alter the bilirubin output transiently but have shown that the alteration is referable merely to a changed rate of pigment evacuation, not to a change in the amount manufactured from day to day, which remains unaffected.[15] Whipple and Hooper collected the bile during 6 or 8 hours of each 24. They made interesting observations on the pigment excreted during this time, following injections of the hemoglobin of laked blood;[16] but the circumstances of the work were such that they could reach no conclusion on quantitative relationships. They noted, however, that in anemic animals the bilirubin output for the 6 hour period was sometimes astonishingly small.

The Calculated Pigment Relationships.

As already stated, every considerable drop in the hemoglobin percentage of our dogs, uninduced by extraneous means, was accompanied by a drop in the bilirubin yield. Granting that a lessened hemoglobin

[11] Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin and Vienna, 4th edition, 1920, i, 746.

[12] Stadelmann, E., Der Icterus und seine verschiedenen Formen. Nebst Beiträgen zur Physiologie und Pathologie der Gallensecretion, Stuttgart, 1891, 40.

[13] Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

[14] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

[15] Rous, P., Broun, G. O., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 421.

[16] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1917, xliii, 258.

percentage means a proportionately lessened amount of the pigment in circulation, one can gain an idea from the data as to whether the known amount of bilirubin excreted during the period in which the blood pigment apparently underwent a lessening represents accurately the amount of blood withdrawn from circulation, or, to speak loosely, destroyed. In Dog 20, for example, the hemoglobin percentage fell from 125 per cent to 86 per cent in the course of 10 days. Does this change find exact expression in the bilirubin output?

Several potential sources of error or complication must be considered prior to an analysis along the lines indicated. Alterations in the hemoglobin percentage are known to be only roughly indicative of changes in the total amount of the pigment. And may not a part of the bilirubin from blood suddenly destroyed be excreted so slowly by the liver as to escape reckoning? The charts furnish evidence on the point. They show that whenever there was a sharp decline in the hemoglobin percentage an equally sharp decline in the bilirubin output took place, both reaching their lowest point at the same time. This could not have happened had bilirubin excretion been notably delayed. The possibility that the substance has other sources besides hemoglobin can, in the present relation, be dismissed. If it has such sources these would appear to be negligible in the present relation, as witness the charts. Changes in the diet influence only the rate of pigment evacuation, and this transiently. But exercise, by increasing the rate of blood destruction, may act to increase markedly the total yield.[3] To minimize this disturbing influence the animals were confined in metabolism cages. Noteworthy errors in bile collection and in bilirubin quantitation were ruled out by the methods employed; and only such figures on hemoglobin percentage have been admitted to consideration as were corroborated by the findings of the preceding or subsequent 24 hours. The factor of blood losses masked by replacement must have influenced the results, sometimes, doubtless, to an important extent. For during the periods supplying the data under analysis, in which the hemoglobin percentage fell, there must have been some repair of the blood loss, since the formation of corpuscles can scarcely have ceased altogether. But for present purposes it will be simplest to suppose that it did cease, and that in consequence the fall in hemoglobin represents the actual blood loss, not, as was actually the case, a somewhat greater loss compensated in part by replacement.

In Table I the actual bilirubin output is contrasted with the expected output during four periods (indicated by brackets and arrows on Text-figs. 2 and 4), in which the hemoglobin percentage lessened rapidly and markedly, without loss from the body of blood, bile pigment, or urobilin, in the stools or urine. Dog 23 of Text-fig. 4 was, like the other animals, in excellent condition at the time, giving no sign of the complicating maladies which rendered its later condition poor. The data on Dog 24 to be found in a previous paper[7] have not been tabulated

TABLE I.

Bilirubin Output during an Intercurrent Fall in the Hemoglobin Percentage (Text-Figs. 2 and 4).

Dog No.	Weight.	Period included.	Calculated blood amount.	Hemoglobin.				Bilirubin output.		Remarks.
				First.	Last.	Drop in hemoglobin.		Expected.	Actual.	
	kg.	*days*	*cc.*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	
23	14½	5	1,334	98	73	25	46.1	1.84	0.42	Bile sterile. No bilirubinuria.
25 a	16	6	1,471	115	100	15	32.3	1.29	0.38	" " " "
25 b		7			91	24	48.8	1.95	0.46	" " " "
20	12¾	10	1,172	125	86	39	63.2	2.53	0.72	" " " "

because the animal occasionally bled from hemorrhoids. The losses in total hemoglobin have been calculated from the percentage changes, on the basis that 9.2 per cent of the body weight of the animal consists of blood;[17] that a blood having 100 per cent of hemoglobin by the Newcomer standard we employed has 13.8 per cent of hemoglobin in every 100 cc.; and that hemoglobin yields 4 per cent by weight of hematin.[18] Most authors assume that hematin appears weight for weight as bile pigment, but in the calculations we have preferred to adopt the conservative estimate of Eppinger and Charnas[19] that for every gram of hematin 0.9 gm. of bilirubin is elaborated.

As the four tabulated instances show (Table I), the actual bilirubin output during periods of blood destruction came to but a fraction of the expected output, averaging about one-third of it, despite the circumstance that several elements in our calculations combined to give a minimal figure for this latter. Corroboratory examples could be culled from our unpublished data.

The Actual Pigment Relationships.

Experiments were now begun to measure the amount of blood destruction more exactly. Advantage was taken of the regularity with which anemia follows upon bile loss, and the total hemoglobin of several animals was ascertained 24 hours prior to intubation of the common duct, and again some days later when the hemoglobin percentage had dropped (Text-fig. 5). The decrease in circulating pigment thus measured must be referred in part to hemorrhage at operation; and though, owing to hemostasis, such part was negligible, it introduces an element of uncertainty into the data. For this reason some later estimates of total hemoglobin were made, to be compared with the findings a few days after operation. But in only one of three instances had any demonstrable loss of hemoglobin occurred in the interval (Text-fig. 5). In order to provide a destruction of corpuscles for the purpose of the observations, recourse was ultimately had to treadmill exercise,[20] and to the transfusion of alien blood.

[17] Smith, H. P., Arnold, H. R., and Whipple, G. H., *Am. J. Physiol.*, 1921, lvi, 336.

[18] The figure for the standard was determined by Dr. J. M. Neill of the Hospital of The Rockefeller Institute, who kindly carried out for us analyses of the pigment content of specimens of dog blood.

[19] Eppinger, H., and Charnas, D., *Z. klin. Med.*, 1913, lxxviii, 387.

[20] Broun, G. O., *J. Exp. Med.*, 1922, xxxvi, 481; 1923, xxxvii, 113.

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				First.	Last.	Drop in hemoglobin.		Expected.	Actual.	
	kg.	*days*	*cc.*	*per cent*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	
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[18] The figure for the standard was determined by Dr. J. M. Neill of the Hospital of The Rockefeller Institute, who kindly carried out for us analyses of the pigment content of specimens of dog blood.

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[20] Broun, G. O., *J. Exp. Med.*, 1922, xxxvi, 481; 1923, xxxvii, 113.

In Tables II and III and Text-figs. 5 and 6, the results of all the experiments find place. The dogs had, without exception, the appearance of vigorous health. They were fed for the most part on a mixture of bread and cooked lean meat in fixed proportion, but occasionally received bread and milk, or raw lean meat. The stools, which were clay-colored, were regularly examined for blood (guaiac reaction) and for stercobilin (Schlesinger's test). Several animals that had been destined for the work were discarded because one or both of the pigments was found. Neither occurred in any of the animals employed. The urine, daily searched for urobilin and urobilinogen and hematoporphyrin, was regularly devoid of the first mentioned substances and of abnormal amounts of the last. Whenever bilirubinuria was sufficiently pronounced for quantitation, the amount of bile pigment escaping in this way was determined according to a procedure already described.[21] Such determinations were necessary in but one animal, Dog 19 (Text-fig. 6). Whenever the bile became infected, incubation tests were carried out to find whether the organisms altered bile pigment. This they never did, nor did they prove pathogenic for the animal harboring them. The dogs were the last of a considerable series intubated for bile collection and fared best of all, the laparotomy wound healing by first intention in practically every case. They were sacrificed after from 1 to 3 months of observation, sometimes because of the development of total obstruction from stone in the common duct, and again for reasons of expediency.

The method of Van Slyke and Salvesen[22] was employed for the determination of total hemoglobin, and by one of us familiarized with it through much previous work.[20] The routine observations on the hemoglobin percentage were made by another individual, and it has been reassuring to observe,—as bearing out an assumption upon which Table I is based,—how nearly the percentage changes in most instances correspond with those in the total pigment. To arrive at a figure on the expected output of bilirubin, the current view on the quantitative relationship between hemoglobin and this pigment was adopted, as it had been for Table I. And, as in the case of this table, the influence of the hematopoietic activities to mask the amount of blood destruction, making it appear less than it actually is, has been left from account. In some instances there was a 24 hour period prior to operation during which, of course, no bile could be collected. The actual bilirubin output for the time can only be conjectured. We have supposed it to equal the largest amount subsequently obtained in the same period of time.

Intercurrent Losses of Total Hemoglobin (Table II, Text-Fig. 5).—In Dog 26 the loss in total hemoglobin during the period of the 6 days following operation, together with the 24 hours immediately preceding it, would seem to have been extremely large,—equivalent to that in

[21] Haessler, H., Rous, P., and Broun, G. O., *J. Exp. Med.*, 1922, xxxv, 533.
[22] Van Slyke, D. D., and Salvesen, H. A., *J. Biol. Chem.*, 1919, xl, 103.

about 500 cc. of whole blood,—despite the fact that hemostasis was nearly perfect. The actual output of bilirubin proved to be less than a third of that warranted by such blood destruction. During the next 6 days the loss of blood was less, though still considerable. It was almost exactly indicated by the changes in the hemoglobin percentage, which had not been the case at first. The actual output of bilirubin now came to one-third of the expected output.

Dog 30 lost during 5 days, inclusive of that before operation, an amount of hemoglobin such as had existed in about 300 cc. of the whole blood. The actual bilirubin yield for the period totalled 43 per cent of the expected quantity. When the total hemoglobin was next determined 10 days later, a more moderate loss in it was found. The actual bilirubin output now slightly exceeded the expected one. It goes without saying that the smaller a hemoglobin loss, and the greater the period of time over which it is distributed, the more important will the factor of cell replacement be, as masking the amount of destruction. Gradual losses may be wholly concealed by replacement. When this is the case the daily bilirubin yield will be considerable, although, since the hemoglobin percentage does not alter, none whatever would be expected under the limiting conditions of our calculation in which the element of replacement is ignored. The findings in the second period of Dog 30, in which the actual bilirubin output exceeded the calculated figure, afford a partial illustration of these facts. They are more strikingly evident in the data on Dog 28.

The hemoglobin percentage of Dog 28 fell abruptly during an initial period of 5 days without any noteworthy diminution in the total hemoglobin. The actual bilirubin output for the period many times exceeded the expected one. An error in the first estimate of total hemoglobin may be invoked to explain the anomalous results, and the discrepancy between the changes in percentage of the pigment and its total quantity point to such a probability. But if the claim be allowed, a similar one must be lodged against the findings for the first period of Dog 26, in which a discrepancy of almost as great dimensions, but opposite in direction, was observed.

During the second period of Dog 28 there was almost no change in the percentage and total quantity of the blood pigment. Whatever the daily loss of cells may have been it was practically concealed by

TABLE II.

Bilirubin Output during Intercurrent Changes in Total Hemoglobin (Text-Fig. 5).

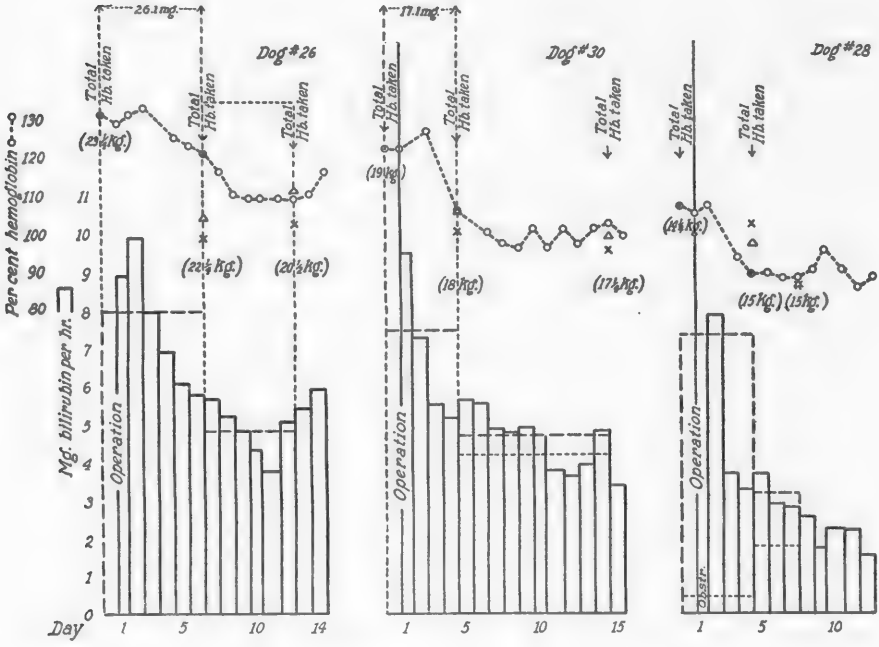
Dog No.	Weight.	Elapsed period.	Cell volume.	Plasma volume.	Blood volume.	Hemoglobin per 100 cc. of blood.	Total hemoglobin.	Loss of hemoglobin within the body.*	Bilirubin output for period.		Remarks.
									Expected.	Actual.	
	kg.	*days*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
											Total hemoglobin determined on the day previous to operation.
	23½		1,121	968	2,089	21.12	441.5				
	22¼	7	1,007	915	1,922	17.2	330.5	105.05	4.38	1.33	After 5 days of bile collection.
26											
	22¼						330.5				
	20½	6	941	837	1,778	15.7	279.0	46.49	1.94	0.69	12th day of bile collection. Bile infected after the 8th day. No bilirubinuria.
											Total hemoglobin determined on the day previous to operation.
	19		990	935	1,925	16.45	316.2				
	18	5	826	925	1,755	14.81	260.0	51.43	2.05	0.88	After 4 days of bile collection.
30											
	18						260.0				
	17¼	10	795	904	1,709	13.67	233.3	22.37	0.90	1.1	13th day after operation. Bile sterile throughout.

28	14½		559	619	1,178	14.83	174.8				Total hemoglobin determined on the day previous to operation.
	15	5	569	642	1,201	13.95	167.8	2.63	0.11	0.73	After 4 days of bile collection.
	15						167.8				
	15	3	543.5	746.5	1,290	12.49	161.0	2.76	0.11	0.22	After 7 days of bile collection. Bile sterile throughout.

* As distinct from the loss incurred in the determinations.

No bilirubinuria in any animal except No. 30, in which an occasional trace was found, as in many normal animals.

replacement. Under such circumstances the actual output of bilirubin could not but greatly exceed the calculated one, as was indeed the case.



X = Hemoglobin per cent as calculated from the reduction in total hemoglobin.

△ = Hemoglobin per cent as calculated from the reduction in total hemoglobin and corrected for change in body weight.

— — — = Actual bilirubin output per hour during the period.

——— = Expected output.

TEXT-FIG. 5. (See Table II.) The actual and the calculated bilirubin yields during periods of intercurrent losses of total hemoglobin.

Induced Losses of Total Hemoglobin (*Table III, Text-Fig. 6*).— Two dogs (Nos. 28 and 30) employed for the work just discussed were exercised some weeks later on a treadmill for 3 to 5 hours on each of 4 successive days, in order to bring about a rapid destruction of red cells. Experience had shown that exercise of the sort could not be

begun immediately after a determination of total hemoglobin by the CO method without great risk of hemorrhage into the tissues through the puncture openings in the jugular veins; while, furthermore, the dogs were not fitted to run well until all the hemoglobin was once again in a condition to combine with oxygen. For these reasons the total blood pigment was ascertained 18 to 24 hours prior to use of the treadmill. Directly after the animal was taken from the mill on the 4th day, the total hemoglobin was again measured, although the 24 hour period of bile collection which included the final exercise hours did not come to an end until the next morning. It follows that the observations upon the blood were not wholly synchronous with those upon the bile but preceded the latter by about 18 hours on the average. Whether one ignore this fact or make allowance for it in computing the actual bilirubin output for the days of exercise,—and we have done both in Table III,—the result is the same. The actual bilirubin output of the animals amounted to little more than half of the expected one.

The destruction of compatible blood transfused to dogs is known to begin early and to proceed rapidly. The course of events in Dog 19, which was somewhat anemic when given 328 cc. of citrated dog blood containing 51.5 gm. of hemoglobin, well illustrates the rule. The concurrent alterations in hemoglobin quantity and bilirubin output proved highly instructive. In a first period of 2 days, following introduction of the strange blood, there was a considerable destruction of cells, as shown by the alterations in total hemoglobin, though without bilirubinuria or symptoms. The increase in the yield of bile pigment was many times less than it should have been on calculation. Blood destruction proceeded more gradually during the next 7 days, as evidenced both by the day to day reductions in the per cent of hemoglobin and the changes in total amount. Bilirubinuria was still absent, and the general condition of the dog excellent. The actual yield of bile pigment for this period was not greatly less than the expected. Now the state of affairs altered almost over night. The hemoglobin percentage began to fall more rapidly, there was pronounced bilirubinuria, and an anemia developed more marked than that prior to transfusion. There was no tissue icterus, but the animal appeared languid. The causes for the developing anemia have been

TABLE III.

Bilirubin Output during the Course of an Induced Reduction in Total Hemoglobin (Text-Fig. 6).

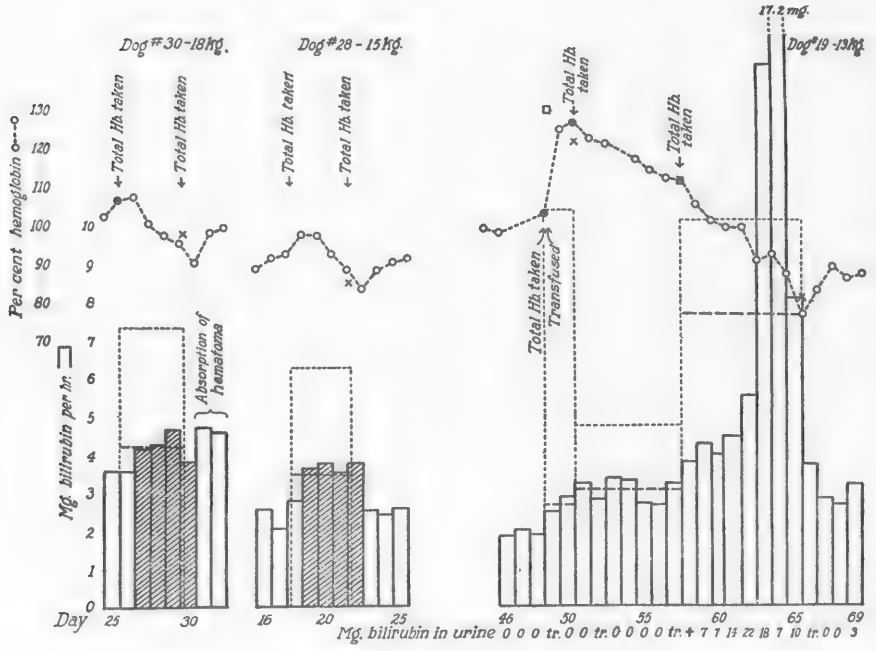
Dog No.	Weight.	Treatment.	Elapsed period.	Cell volume.	Plasma volume.	Blood volume.	Hemoglobin per 100 cc. of blood.	Total hemoglobin.	Loss of hemoglobin within the body.*	Bilirubin output for period.		Remarks.
										Expected.	Actual.	
	kg.		*days*	*cc.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
30	18	5 hrs. of treadmill exercise on each day except the last, when 3 hrs. only was given.		821	1,024	1,845	13.95	257.4				The total hemoglobin was determined 22 hrs. before beginning exercise, and again 3 hrs. after its close. No bilirubinuria. Bile sterile.
			4	765	1,185	1,950	12.10	236.0	17.4	0.7	0.4 (0.4)†	
28	$14\frac{3}{4}$	5 hrs. of treadmill exercise on each day except the last, when 4 hrs. only was given.		531	669	1,200	14.1	169.2				The total hemoglobin was determined 22 hrs. before beginning exercise, and again 3 hrs. after its close. No bilirubinuria. Bile infected.
			4	511.5	704.5	1,216	12.35	150.1	15.05	0.6	0.33 (0.35)†	

	13	Transfused.		564	598	1,162	15.8	183.6			The total hemoglobin was determined 2 hrs. before transfusion.
			Calculated amount of hemoglobin after transfusion 232.1 gm.								
			2	656	483	1,139	19.11	217.6	12.58	0.5	0.11	No bilirubinuria. Bile infected.
19	13¼		7	602	532	1,134	16.94	217.6 192.2	19.89	0.8	0.505	No bilirubinuria.
			8	492	706	1,198	11.58	192.2 138.9	48.75	1.95	1.57	Marked bilirubinuria (the 85 mg. of pigment excreted during the period are included in the actual bilirubin output).

* As distinct from the loss incurred in the determinations.

† Total bilirubin output during the four 24 hour periods of bile collection which cover the days on which the dogs were exercised.

dealt with by Robertson.[9] Prominent among them is marrow inactivity induced by the plethora after transfusion. Because of the temporary lack of need for new red cells, there occurs almost no elaboration of them by the animal while the plethora lasts, and when the strange blood is suddenly made away with through the action of induced antibodies, anemia inevitably develops because the residuum of host corpuscles is small. The expected bilirubin output during


Dog #30-18 Kg.
Dog #28-15 Kg.
17.2 mg.
Dog #19-13 Kg.
Total Hb taken
Per cent hemoglobin
Mg. bilirubin per hr.
Absorption of hematoma
Total Hb taken
Transfused
Day
Mg. bilirubin in urine 0 0 0 tr. 0 0 tr. 0 0 0 0 tr. + 7 7 15 22 18 7 10 tr. 0 0 3

X = Hemoglobin per cent as calculated from the reduction in total hemoglobin.

□ = Hemoglobin per cent as calculated from the amount of hemoglobin added by transfusion.

——— = Actual bilirubin output per hour during the period.

------- = Expected output.

TEXT-FIG. 6. (See Table III.) The actual and the calculated bilirubin yields during periods of induced loss of total hemoglobin. The actual output on the days of exercise finds record in the cross-hatched columns.

this period of rapid destruction in Dog 19 was large, more than twice that of the days immediately preceding, yet the actual output came nearer to equalling it than previously. Thereafter an abrupt turn for the better in the general condition was noted, the bilirubinuria ceased, the bile became much less pigmented, and recovery from the anemia began. The total amount of hemoglobin was not ascertained again.

DISCUSSION.

In the analysis (Tables II and III) of the data from the last two sets of experiments here discussed, the actual bilirubin output has been contrasted with the expected one which was estimated from the known loss in total hemoglobin as if so much pigment in the form of blood had been taken from a jar and bilirubin derived from it by chemical means. As already mentioned, losses masked by replacement through the hematopoietic activities were disregarded, and of necessity, since they could not be gauged. Despite their potential influence to increase the bilirubin output, this was always less, usually several times less, than the expected quantity whenever a marked and rapid loss of the blood pigment was demonstrable.

These observations show clearly that the quantitative relation existing between blood destruction and the bilirubin output is very different from that generally supposed. Save under special circumstances, which themselves go to prove the rule, the yield of bile pigment was far below what it should have been, to judge from the amount of hemoglobin removed from the circulation. Under the special circumstances referred to, when the body was oversupplied for the time being with the products of blood destruction, as shown by the state of the urine, the actual yield of bilirubin approached the calculated one. Usually the actual yield was less than half of this latter, which was computed on a basis that yielded a minimal figure for it.

Why, in our experiments, did the actual yield of bilirubin fall so far short of the expected one? The quantitative relationships between hemoglobin, hematin, and bilirubin, as such, can scarcely be impeached. Nor do the charts afford indications of any delay in the excretion of bile pigment. Other sources of it there may be besides the blood, but, as has been said, these could have no influence under the conditions of the work. The explanation evidently is that a

part of the pigment from destroyed blood, out of which bilirubin can be formed, undergoes retention within the body,—a possibility suggested by Stadelmann many years ago.[12] That the iron-containing derivatives of the hemoglobin molecule are held for future use has been generally acknowledged. Bilirubin has been thought of as waste stuff. But recently we have brought evidence that some of it is reabsorbed after reaching the intestine.[6] Whether there is a salvage of material by this means for later use in the manufacture of new red cells remains to be determined. But the blood and the bile changes which follow upon loss of the bile must be discussed in the light of such a possibility.

An immediate question in connection with the postoperative blood changes recorded in the present paper is whether they are referable to the loss of pigment in the bile. No other cause for them was evident if one except a not infrequent reddening of the duodenum, unaccompanied by hemorrhage and consequent doubtless on the absence of bile. Anemia developed in every one of eleven animals studied with special reference to the point, most of them extremely vigorous and all kept under conditions and on a diet which suffice in normal dogs for the maintenance of an excellent blood state. The hemoglobin percentage of Whipple and Hooper's long tended fistula dogs remained consistently high.[13] The observations of these authors appear to have been begun a fortnight or more after operation, when the fistula wound had healed. They attribute the well-being of their dogs to liver feedings or the intermittent passage of a little bile into the intestine through some narrow channel in the tissues. The animals had access to the external fistula opening during 16 or 18 hours of each 24 and may well have licked up some of the escaping bile,—though that they obtained much of it seems unlikely.[23] Bile deprivation in our dogs was complete. Furthermore, ours were kept caged because of the influence of exercise upon the bilirubin output, whereas Whipple and Hooper's were allowed to run in a yard daily. Exercise, as Broun has shown,[20] stimulates the hematopoietic tissue. Possibly it is responsible for alterations in hemoglobin metabolism that are sufficient to counteract the tendency to anemia after bile loss. How-

[23] Foster, M. G., Hooper, C. W., and Whipple, G. H., *J. Biol. Chem.*, 1919, xxxviii, 367.

ever this be, the present findings leave no doubt that such a tendency exists; and the demonstrated activity of the body to conserve bile pigment, together with its ability to retrieve a portion of that excreted into the intestine, give reason for the belief that the anemia results from bilirubin loss. Human beings with a bile fistula frequently develop anemia—in one case reported by Balfour and Ross[24] the hemoglobin had fallen to 30 per cent after 3 years of bile loss, although the physical condition of the individual was otherwise good. But many patients losing all the bile fail rapidly and are the subject of complicating disturbances to which such blood changes as occur may well be referable.

The rapid falling off in the bilirubin output during the 1st week after operation, or, to put the case more directly, the high postoperative yield as compared with the later one, is in some instances clearly the result in part of the destruction of red cells extravasated at operation, as we have pointed out. Possibly some corpuscles are injured by the anesthetic. In any event, the destruction within the body of but a few extra cubic centimeters of blood would suffice, according to current views, to swell markedly the bilirubin total of a 24 hour period. But there is another potential reason for the high output of the 1st day after operation. Under normal circumstances the bilirubin yield by the liver may represent not merely excretion of the pigment but an enterohepatic circulation of it.[6] With the sudden elimination of the latter factor, on intubation of the animal, the bilirubin yield should fall off.

The postoperative anemia in intubated dogs,—one feature of which is a low color index of the cells,—develops a little later and more slowly than the fall in the bilirubin output. It can be explained, as has just been indicated, by the continued loss of such part of the bile pigment as would normally be saved from the intestine by reabsorption and utilized in the formation of new corpuscles. In this relation the evidence in our experiments for differing degrees of conservation of the pigment from destroyed blood has significance. In the transfused dog, No. 19 (Table III, Text-fig.6), which received alien blood when itself anemic, almost all the pigment derived from

[24] Balfour, D. C., and Ross, J. W., *Arch. Surg.*, 1921, iii, 582.

the destruction of the 2 days immediately following transfusion was retained, to judge from the bilirubin output. During the next period, with a continuance of blood destruction, the actual yield of bile pigment came closer to the expected one; and in a final period, when the latter was large, approximated it still more nearly. The course of events was exactly what one would have anticipated had the body at first a need for the pigment, hence retaining most of it, and then gradually been supplied with as much and more than was required. In line with this idea of a conserving mechanism, which may on occasion be active in saving pigment or again lack occasion to function, is the fact that in those of our animals in which blood destruction was precipitated by exercise or transfusion, the actual yield of pigment in the bile more nearly approximated the expected one than when the reduction in hemoglobin was not artificially induced. The conditions may well have been less favorable to pigment conservation in the first mentioned series of instances. Whipple and Hooper record experiments showing that in anemic dogs given injections of small quantities of hemoglobin, the bilirubin increase during the next 6 hours was far less than in similarly treated non-anemic animals.[16] They did not follow the changes to completion, but their protocols suggest a pigment conservation in the presence of body need. There was no evidence for this in the few protocols of Brugsch and Yoshimoto,[25] who concluded that injected hematin was quantitatively represented in the bilirubin output. But much, obviously, will depend on the immediate body need and the amount of pigment given. Brugsch and Retzlaff injected it on many consecutive days, and lumped their findings for the period.

Consecutive observations, extending over many weeks, on the hemoglobin percentage and the bilirubin output (see Text-fig. 4; also McMaster, Broun, and Rous[8]) bring out the presence of a relationship between the two which, on cursory inspection, would appear to be undisturbed by normal influences other than exercise. But there are hidden factors which act to distort this relationship. Whenever the hemoglobin percentage is stationary or rising, such blood destruction as may be going on is, of course, wholly concealed through the hema-

[25] Brugsch, T., and Yoshimoto, *Z. exp. Path. u. Therap.*, 1910–11, viii, 639.

topoietic activity; and when the percentage falls gradually, the concealment continues, though now it fails to mask completely the destructive process. Under such circumstances the bilirubin yield cannot be an accurate reflection of the percentage alteration. Furthermore, the amount of it put out in the bile will be directly affected by variations in the degree of pigment conservation. It is remarkable that a daily yield which is the resultant of blood changes that are partially concealed and of a pigment conservation which may vary should so nearly reflect gross alterations in hemoglobin as indicated by the percentage value of blood specimens. If one chooses to think not in terms of this latter substance but in those of the cells in which it is lodged, and ignores intercurrent changes in the color index, then the bilirubin yield becomes a more or less accurate expression of cell mortality. On first view it appears to be a strikingly faithful one. When the cell population is large or small, the number of cell deaths should, other things being equal, likewise be large or small, and so too with the bilirubin output. Such is the actual case. Indeed, the variations in output so closely follow those in the hemoglobin percentage as to give the impression that cell deaths are registered practically at once, that is to say within a few hours, in terms of bile pigment. But always one has to reckon with masked blood destruction and pigment conservation as disturbing factors. Here and there in the charts their influence is manifest. Whenever a noteworthy remission from anemia occurred, the increase in cell number evidenced by the mounting per cent of hemoglobin involved, by corollary, an increase in the eventual number of cell deaths. That this number increased even while the repair was going on may be inferred from the rise in the bilirubin yield. But here a curious fact may be noted. The rise did not parallel but actually preceded that in the hemoglobin per cent. Either, as repair began, more cells were destroyed than ordinarily, or else the need for bilirubin conservation had lessened. As a matter of fact, proof has been brought in a previous paper of a special corpuscular wastage during recovery from anemia,[26] traceable to the circumstance that many of the cells placed in circulation by the hematopoietic tissue are unfit to withstand the exigencies of life there. It will further

[26] Robertson, O. H., and Rous, P., *J. Exp. Med.*, 1917, xxv, 665.

be seen from the charts that whenever a marked drop in the hemoglobin percentage occurred, uninduced by artificial aids, the bilirubin yield fell simultaneously, but generally to a much more marked extent. The factor of replacement must have been of relatively slight importance at such periods, since the marrow was inactive as proven by the small number of circulating reticulocytes. In four specimen instances (see Text-fig. 4; also the curve in a previous paper[8]) the hemoglobin dropped from 118 per cent to 80 per cent, 99 per cent to 70 per cent, 92 per cent to 76 per cent, and 93 per cent to 62 per cent, whereas the bilirubin decreased from 6.2 mg. to 3 mg. (118 per cent to 57 per cent), 4.4 mg. to 2.2 mg. (99 per cent to 46 per cent), 5.3 mg. to 2.2 mg. (92 per cent to 38 per cent), and 5 mg. to 2.9 mg. (93 per cent to 54 per cent) per hour respectively. An increasing conservation of pigment as the anemia gradually developed would explain these findings.

Instances of the sort referred to, in which the drop in the hemoglobin percentage must have come about in the main through a progressive depletion of the current stock of cells without adequate replacement, and in which the bilirubin output decreased concurrently as a consequence of the lessening of cell population with, by corollary, a lessened number of cell deaths from "natural causes," may be contrasted with those others in which a decrease in blood pigment was induced by extraneous influences (Text-figs. 5 and 6). Here the bilirubin yield mounted while the anemia was developing, as would follow from the excessive mortality in a thinning population of corpuscles. It is evident that the changes in the bilirubin output of intubated animals from day to day should aid one to tell whether a given anemia is the expression of ordinary wear and tear on the corpuscles in the lack of the usual cell replacement, or whether, on the other hand, it results from increased blood destruction despite what the hematopoietic tissue may be doing in the way of replacement.

SUMMARY.

In dogs intubated for the collection of all of the bile, a marked falling off in the yield of bilirubin is regularly to be noted after operation, followed soon by an anemia of secondary character. Though, in the absence of complications, the anemia is mild, it persists despite the

excellent general condition of the animal. Intercurrent changes in the hemoglobin percentage take place from time to time, and these are accompanied by very similar fluctuations in the bilirubin quantity. At first inspection the data strongly suggest that blood destruction finds accurate quantitative expression in the yield of bile pigment. But this is not the case. True, the destruction finds expression in terms of bile pigment and practically at once; and the data support the conception that bilirubin has no other sources besides the hemoglobin of destroyed blood. But our experiments show that the amount of it put forth during the development of an anemia from gradual blood destruction, either intercurrent or induced, is far below that derivable from the net quantity of hemoglobin disappearing from the circulation. The discrepancy is referable to a process of pigment conservation which varies in proportion to the body need.

We have shown in a previous paper that bile pigment may on occasion be absorbed from the intestinal tract. This fact and those reported in the present communication lead one to question the accepted view that bilirubin is mere waste material eliminated by way of the liver. The anemia which develops upon total loss of the bile is not improbably consequent on the pigment loss therewith.

Current methods of computing the rate of normal and pathological blood destruction from the bilirubin (or urobilin) yield are unsound in principle and open to large error in practise. Yet there is no doubt that day to-day variations in the output of bile pigment result from changes in the blood; and on occasion they provide enlightening evidence of the nature of hemic events.

ACTION OF SERUM ON FIBROBLASTS IN VITRO.

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I.

INTRODUCTION.

It had been observed previously that fragments of connective tissue, cultivated in plasma, did not increase in mass in spite of the activity they displayed in their medium. The growth of fibroblasts was no more extensive in serum than in media containing a small amount of serum or no serum at all, even when embryonic tissue juice was added to the medium.[1] Under the conditions of the experiments, serum never increased cell activity. As the action of serum had been determined merely by the rate of cell migration during a short time,[1] it was necessary to ascertain whether the duration of life of pure cultures of fibroblasts was modified by the presence of serum in varied dilutions and by its omission.

II.

EXPERIMENTAL.

The tissues were obtained from a 10 year old strain of fibroblasts,[2] and the serum from the plasma of young adult chickens which had fasted for 48 hours. The fibroblasts were cultivated in a medium of fibrinogen suspension,[3] Tyrode solution, and in varied amounts of serum. The medium was modified in different ways by substituting for Tyrode solution the requisite amounts of serum or tissue juice, according to the nature of the experiments. In order to prevent

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

[2] Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

the digestion of the fibrin in a serum-free medium, a small amount of egg yolk was added. Coagulation of the fibrinogen suspension was brought about by embryonic tissue juice in a concentration of 1:200. The composition of the medium was as follows:

Fibrinogen suspension	1 volume.
Doubly concentrated Tyrode solution containing 1 per cent egg yolk	1 volume.
Tyrode solution containing 1:50 embryonic tissue juice	1 volume.
Tyrode solution	1 volume.

TABLE I.

Influence of 0 Per Cent and 7 Per Cent Serum on the Duration of Life of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase. Tyrode solution.	Experiment 1. Relative increase. Serum.	Experiment 1. Ratio: $\frac{S}{T}$.	Experiment 2. Relative increase. Tyrode solution.	Experiment 2. Relative increase. Serum.	Experiment 2. Ratio: $\frac{S}{T}$.	Experiment 3. Relative increase. Tyrode solution.	Experiment 3. Relative increase. Serum.	Experiment 3. Ratio: $\frac{S}{T}$.	Experiment 4. Relative increase. Tyrode solution.	Experiment 4. Relative increase. Serum.	Experiment 4. Ratio: $\frac{S}{T}$.
1	340	3.38	3.28	0.97	3.53	3.38	0.96	3.93	4.06	1.03	3.67	3.46	0.94
2	351	2.12	2.62	1.23	2.74	2.33	0.85	2.33	2.37	1.02	2.27	2.23	0.98
3	366	1.59	1.60	1.00	2.13	1.78	0.84	2.23	2.04	0.92	1.90	1.83	0.96
4	376	1.40	1.50	1.07	0	1.85		0	1.80	0	0	1.34	
5	386	1.00	1.12	1.12		0.85			0			0	
6	398	0	0.47	0		0							

Passage No.	Culture No.	Experiment 5. Relative increase. Tyrode solution.	Experiment 5. Relative increase. Serum.	Experiment 5. Ratio: $\frac{S}{T}$.	Experiment 6. Relative increase. Tyrode solution.	Experiment 6. Relative increase. Serum.	Experiment 6. Ratio: $\frac{S}{T}$.	Experiment 7. Relative increase. Tyrode solution.	Experiment 7. Relative increase. Serum.	Experiment 7. Ratio: $\frac{S}{T}$.	Experiment 8. Relative increase. Tyrode solution.	Experiment 8. Relative increase. Serum.	Experiment 8. Ratio: $\frac{S}{T}$.
1	325	3.62	3.80	1.05	4.21	4.21	1.00	3.25	3.69	1.13	3.60	3.00	0.83
2	339	3.48	3.55	1.02	3.29	3.50	1.06	3.03	3.13	1.03	2.69	2.82	1.05
3	350	2.13	2.21	1.04	2.00	2.00	1.00	1.54	1.58	1.03	1.69	1.48	0.87
4	365	1.89	1.66	0.88	1.81	1.72	0.95	1.73	1.62	0.94	1.78	1.62	0.91
5	375	1.70	1.50	0.88	1.60	1.00	0.63	0	0.53	0	2.00	1.85	0.93
6	385	0	0	0	1.50	0	0				1.50	1.30	0.87
7											0	0	0

The medium was modified by adding serum or tissue juice to replace a similar amount of Tyrode solution. The pH of Tyrode solution was 8.0, that of serum and tissue juice from 7.0 to 7.6, and that of fibrinogen suspension 6.8.

TABLE II.

Influence of 0 Per Cent and 25 Per Cent Serum on the Duration of Life of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase. Tyrode solution.	Experiment 1. Relative increase. Serum.	Experiment 1. Ratio: $\frac{S}{T}$.	Experiment 2. Relative increase. Tyrode solution.	Experiment 2. Relative increase. Serum.	Experiment 2. Ratio: $\frac{S}{T}$.	Experiment 3. Relative increase. Tyrode solution.	Experiment 3. Relative increase. Serum.	Experiment 3. Ratio: $\frac{S}{T}$.	Experiment 4. Relative increase. Tyrode solution.	Experiment 4. Relative increase. Serum.	Experiment 4. Ratio: $\frac{S}{T}$.
1	381	3.30	3.35	1.01	2.95	3.05	1.03	2.80	3.00	1.07	2.66	3.20	1.20
2	392	2.55	2.06	0.81	2.60	2.15	0.82	2.45	1.38	0.56	2.20	1.84	0.84
3	402	1.60	1.57	0.98	2.50	1.77	0.70	1.17	0	0	0.78	0.50	0.64
4	409	1.20	0.30	0.25	1.22	0.50	0.41	0			1.80	0.29	0.16
5	415	1.74	0	0	1.53	0	0				0	0	0
6	424	0			0								

Passage No.	Culture No.	Experiment 5. Relative increase. Tyrode solution.	Experiment 5. Relative increase. Serum.	Experiment 5. Ratio: $\frac{S}{T}$.	Experiment 6. Relative increase. Tyrode solution.	Experiment 6. Relative increase. Serum.	Experiment 6. Ratio: $\frac{S}{T}$.	Experiment 7. Relative increase. Tyrode solution.	Experiment 7. Relative increase. Serum.	Experiment 7. Ratio: $\frac{S}{T}$.
1	388	2.75	2.46	0.90	2.82	2.34	0.83	3.00	3.25	1.08
2	397	1.83	2.10	1.15	2.50	2.56	1.02	2.03	2.30	1.13
3	404	1.80	0.67	0.37	1.40	1.50	1.07	1.57	1.57	1.00
4	410	1.72	0	0	1.68	0.44	0.26	1.24	0.22	0.18
5	416	0			1.58	0.41	0.26	0.90	0.20	0.22
6	423				0	0	0	0	0	0

The tissues were incubated for 48 hours, and the rate of growth was measured in the ordinary manner.[4] Then they were transferred to a new medium of the same composition and the operation was repeated until death occurred, or until the rates of growth of fibroblasts in

[4] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

both experimental and control media had been compared during a number of passages.

1. Duration of Life of Fibroblasts in Media Containing 0 Per Cent and 7 Per Cent Serum.—Two groups of four experiments each were made. The results are averaged in Table I. The fragments of tissue in 0 per cent and 7 per cent serum grew at about the same rate. The fibroblasts migrated actively into the medium after each transfer. Nevertheless, the mass of the tissues never increased. After five or six passages, death occurred (Table I).

2. Duration of Life of Fibroblasts in Media Containing 0 Per Cent and 25 Per Cent Serum.—The first group was composed of four and the second of three experiments (Table II). The duration of life of the fibroblasts was slightly shorter in 25 per cent serum than in 0 per cent.

3. Duration of Life of Fibroblasts in Media Containing 50 Per Cent Serum.—The duration of life of fibroblasts in 50 per cent serum was compared to that in 25 per cent. In 25 per cent serum, death occurred after the fifth or sixth passage; in 50 per cent serum, after the fifth passage (Table III).

4. Rate of Growth of Fibroblasts in Media Containing 0 Per Cent and 10 Per Cent Serum in the Presence of 25 Per Cent Embryonic Tissue Juice.—The mass of the tissues increased, and the cultures had to be divided. But the rate of growth was no greater in 10 per cent serum than in the media containing no serum (Table IV). As a rule, the migration of the cells was slightly more active in the serum-free medium. As the life of fibroblasts in media containing embryonic tissue juice may be very long, and possibly indefinite, the condition of the tissues in both media was compared after ten passages. The average ratio of the rate of growth of fibroblasts in 0 per cent and 10 per cent serum was 0.96. There was, then, no difference between the tissues cultivated in media containing 0 per cent and 10 per cent serum, in the presence of embryonic tissue juice.

5. Rate of Growth of Fibroblasts in Media Containing 0 Per Cent and 25 Per Cent Serum in the Presence of 25 Per Cent Embryonic Tissue Juice.—The experiments summarized in Table V indicate that the presence of 25 per cent serum failed to increase the rate of growth of fibroblasts.

TABLE III.

Influence of 25 Per Cent and 50 Per Cent Serum on the Duration of Life of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1.			Experiment 2.			Experiment 3.		
		Relative increase.			Relative increase.			Relative increase.		
		25 per cent serum.	50 per cent serum.	Ratio: $\frac{50 \text{ per cent}}{25 \text{ per cent}}$.	25 per cent serum.	50 per cent serum.	Ratio: $\frac{50 \text{ per cent}}{25 \text{ per cent}}$.	25 per cent serum.	50 per cent serum.	Ratio: $\frac{50 \text{ per cent}}{25 \text{ per cent}}$.
1	311	4.00	3.00	0.75	3.54	2.54	0.72	4.09	3.00	0.73
2	326	3.24	2.44	0.75	2.78	2.08	0.75	3.06	2.29	0.75
3	338	3.07	2.29	0.75	2.38	1.70	0.71	2.70	1.86	0.69
4	349	1.67	1.06	0.63	1.05	0.68	0.65	1.55	0.89	0.57
5	364	1.63	0.37	0.23	1.18	0	0	1.21	0.36	0.30
6	374	0.64	0		0			0	0	0

TABLE IV.

Influence of 0 Per Cent and 10 Per Cent Serum in the Presence of 25 Per Cent Tissue Juice on the Rate of Growth of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1.			Experiment 2.			Experiment 3.			Experiment 4.		
		Relative increase.			Relative increase.			Relative increase.			Relative increase.		
		Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.	Tyrode solution.	Serum.	Ratio: $\frac{S}{T}$.
1	370	4.15	4.00	0.96	3.29	3.44	1.05	3.47	2.73	0.79	3.70	3.49	0.94
2	377	3.36	3.65	1.09	2.93	3.23	1.10	2.90	3.48	1.20	3.25	3.40	1.05
3	387	2.60	2.20	0.85	3.15	2.72	0.86	2.66	2.90	1.09	2.44	2.96	1.21
4	396	1.77	1.87	1.06	1.70	2.30	1.35	1.80	2.15	1.19	2.17	2.42	1.12
5	403	2.17	1.70	0.78	1.83	1.59	0.87	1.60	1.69	1.06	1.75	1.40	0.80
6	408	3.20	2.03	0.63	4.13	2.58	0.62	3.44	3.08	0.90	4.73	3.08	0.65
7	414	6.68	4.92	0.74	4.74	4.85	1.02	5.24	5.80	1.11	5.30	4.15	0.78
8	425	7.78	6.43	0.83	4.70	4.67	0.99	4.73	4.46	0.94	5.66	5.08	0.90
9	429	4.80	4.50	0.94	4.65	4.30	0.92	3.83	4.16	1.09	3.50	3.46	0.99
10	432	3.37	3.45	1.02	4.25	4.30	1.01	3.89	3.89	1.00	4.04	3.62	0.90

III.

DISCUSSION.

The duration of life of fibroblasts was found to be practically identical in media containing no serum and 7 per cent serum. It was slightly shortened by the addition of 25 per cent serum, and still more so by 50 per cent serum. Under the conditions of the experiments, serum proteins were apparently not utilized by the fibroblasts. These results confirmed the information obtained from previous experiments[1] in which the influence of serum on the activity of fibroblasts

TABLE V.

Influence of 0 Per Cent and 25 Per Cent Serum in the Presence of 25 Per Cent Tissue Juice on the Rate of Growth of Homologous Fibroblasts.

Passage No.	Culture No.	Experiment 1. Relative increase. Tyrode solution.	Experiment 1. Relative increase. Serum.	Experiment 1. Ratio: $\frac{S}{T}$.	Experiment 2. Relative increase. Tyrode solution.	Experiment 2. Relative increase. Serum.	Experiment 2. Ratio: $\frac{S}{T}$.	Experiment 3. Relative increase. Tyrode solution.	Experiment 3. Relative increase. Serum.	Experiment 3. Ratio: $\frac{S}{T}$.
1	433	3.10	3.24	1.05	3.14	3.27	1.04	3.82	3.54	0 93
2	444	4.23	4.50	1.06	4.40	4.52	1.03	4.00	4.30	1.08
3	454	3.46	3.25	0.94	3.35	3.34	1.00	4.32	3.65	0.84
4	466	3.45	2.90	0.84	3.85	3.85	1.00	4.50	4.05	0.90
5*	482	1.41	1.50	1.06	1.74	1.83	1.05	No measurement.		
6	491	3.54	3.43	0.97	4.40	4.35	0.99	4.56	4.56	1.00
7	498	4.15	3.80	0.92	4.15	4.28	1.03	4.15	4.63	1.12
8	513	2.95	3.10	1.05	No measurement.			No measurement.		
9	530	3.14	3.20	1.02	4.10	4.04	0.99	3.39	3.61	1.06

* Cultures divided, one-half discarded. Tracings made after 24 hours growth.

was studied during a short time. After ten passages in the presence of embryonic tissue juice, fibroblasts were as active in Tyrode solution alone as in Tyrode solution containing a small amount of serum. Their rate of growth was slightly decreased when serum was present in a higher concentration.

Serum, therefore, cannot be considered a culture medium in the proper sense of the word. No new protoplasm is built up by the cells from substances contained in the medium. The residual activity,

manifested by the cells in media composed only of plasma, serum, or Tyrode solution, is due to substances stored in the tissues or the cells themselves. The failure of the fibroblasts *in vitro* to utilize the serum proteins confirms the observation made by several experimenters[5] that tissues cultivated in plasma do not increase in mass, and that cell multiplication may be due to a mere transfer of food material from the central part of the tissue fragment to the periphery. As soon as embryonic tissue juice is added to the medium, the mass of the tissues increases rapidly. But the presence of serum under low or high concentration does not cause the production of a larger amount of tissue. The new protoplasm is made from substances contained in the embryonic tissue juice and not in the serum. Serum proteins are not used by the fibroblasts as food material. They appear merely to decrease the rate of cell multiplication.

IV.

CONCLUSIONS.

It may be concluded that, under the conditions of the experiments:

1. The duration of life of fibroblasts is not altered by the presence of 7 per cent serum in a medium composed of fibrin and Tyrode solution, but is slightly decreased when the concentration of the serum reaches 25 per cent.

2. Fibroblasts cultivated in serum or in Tyrode solution are only in a condition of survival; they do not build up new protoplasm from the serum proteins and their mass does not increase.

3. When embryonic tissue juice is added to the medium, the tissues increase in mass. But the rate of growth is the same in media containing 0 per cent and 10 per cent serum. In 25 per cent serum, however, the rate of growth slightly decreases. Even in the presence of embryonic tissue juice, serum does not increase the rate of growth of connective tissue.

4. The nitrogenous compounds contained in serum are not used as food material by fibroblasts growing *in vitro*.

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277. Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421. Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

SEPARATION OF THE TOXINS OF BACILLUS DYSENTERIÆ SHIGA.

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In 1920, Olitsky and Kligler[1] separated the poison of *Bacillus dysenteriæ* Shiga into two physically and biologically distinct toxins. One, a true, soluble, exotoxin, an early product of the growth of this microorganism *in vitro*, is relatively heat-labile and yields an antiexotoxic immune serum. The other, an endotoxin, a product of the autolysis, or disintegration, of the bacillus with the resultant liberation of intracellular components, is heat-stable and is not neutralized by antiexotoxic serum. The exotoxin has a specific affinity for the central nervous system in the rabbit; the endotoxin, on the other hand, affects only the intestinal tract.

Since the exotoxin arises in the early period of growth, and since the endotoxin results from bacterial disintegration—a factor difficult to control in growing cultures—there is great technical difficulty in preparing pure endotoxin or exotoxin directly from the Shiga bacillus. Olitsky and Kligler have indicated methods for their separation. In this paper we shall describe an additional method which we believe to be more simple and effective. In this way the previous observation that the toxins of this microorganism are distinct has been confirmed.

It is relatively more simple to obtain pure neurotoxic exotoxin. Merely the filtrate of the early growths of Shiga bacilli, before bacterial disintegration occurs, suffices. Hence young, vigorously growing, well oxygenated cultures, incubated at 37°C. for from 1 to 3 or even to 5 or 7 days, depending on the rate of autolysis, or disintegration, when filtered through Berkefeld V or N candles, yield pure exotoxin in the bacteria-free filtrate.[1] This is not the case, however, with

[1] Olitsky, P. K., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxi, 19.

endotoxin. The endotoxin which arises later as a result of bacterial dissolution is always admixed with the exotoxin which is first produced in the medium, and therefore the toxins require mechanical separation for purification.

Suppression of Exotoxin by Anaerobiosis.

If the exotoxic function of Shiga bacilli could be suppressed, the problem of producing pure endotoxin directly from disintegrated bacilli would be simplified. In seeking for a method for this suppression, use was made of the underlying principle of rapid and increased exotoxic production—oxygenation. Roux and Yersin,[2] by passing a stream of oxygen through the cultures or employing thin layers of culture fluid, determined the requirements for the prompt and effective production of diphtheritic soluble toxin—a method subsequently yielding successful results in the hands of Park and Williams[3] and accepted generally as a standard, not only for the diphtheritic exotoxin but also for that of other bacteria, including the Shiga bacillus.[1,4] If the converse held, that is, if Shiga bacilli were grown in an oxygen-free atmosphere and in a deep layered medium, the exotoxic function would be lowered or destroyed so that mechanical disintegration of the bacterial bodies in this medium might yield pure endotoxin.

In 1904, Rosenthal[4] found that anaerobic conditions diminish the yield of Shiga bacillus poison, although no mention was made of which type of the toxin is involved. Later, Doerr[5] affirmed that under similar conditions no specific dysenteric toxin is present in filtrates. No reference was made by these investigators, however, to any toxin and its type resident in the bacterial bodies comprising the residuum.

Experiments with Aerobic Cultures.

Our first attempts at experimentation related to the study of the toxicity of the particular strain of *Bacillus dysenteriæ* Shiga employed,

[2] Roux, E., and Yersin, A., *Ann. Inst. Pasteur*, 1888, ii, 629; 1889, iii, 273.

[3] Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 7th edition, 1920, 342.

[4] Rosenthal, L., *Deutsch. med. Woch.*, 1904, xxx, 235.

[5] Doerr, R., Das Dysenterietoxin, Jena, 1907, 30.

Strain 109.[1] This culture was maintained in artificial media for $5\frac{1}{2}$ years, or $3\frac{1}{2}$ years after having been tested by Olitsky and Kligler.[1]

Experiment 1.[6]—2 per cent peptone broth was placed in a Noguchi leptospira vaccine flask—a flask made of Pyrex glass measuring 10 cm. in height, with a diameter at the neck of 1.5 cm. and at the base of 7.5 cm. The object in using this sort of vessel was to allow thorough aeration, by providing a large surface area. Sufficient medium was employed to make a layer of broth 0.5 cm. deep. The broth was adjusted to pH 7.4. We desired to avoid an alkaline reaction since it encourages autolysis, or disintegration, of the bacilli, thus liberating the intracellular endotoxin. The medium was inoculated with a loopful of *B. dysenteriæ* Shiga, Strain 109, obtained from a plain agar slant growth of 18 hours duration. The flask was incubated for 3 days at 37°C. and frequently shaken to aerate the contents. The culture was then centrifuged and the supernatant fluid decanted and filtered through Berkefeld V candles. The bacteria-free filtrate was injected into the auricular vein of rabbits weighing about 1,800 gm.

The rabbits which received 1, 2, 5, and 10 cc. died, depending on the dosage, within 5 to 24 hours, with symptoms indicative of lesions in the medulla and upper cervical cord. These lesions were noted in stained sections and consisted of extensive hemorrhages, chiefly, along with edema, necrosis, and changes in neurons already described by Olitsky and Kligler.[1] A rabbit injected with 0.4 cc. showed no symptoms until the 4th day after injection, when complete paralysis occurred in the anterior extremities with paresis of the posterior limbs, and complete paralysis of all the extremities on the 5th day. The nervous symptoms endured for 4 days; thereafter the animal returned slowly to normal. A rabbit injected with 0.35 cc. of the filtrate exhibited slight weakness of the posterior extremities 4 days after inoculation, which lasted for 1 day only. In none of all these rabbits injected with the filtrate were any intestinal symptoms noted.

At the same time, the centrifuged, sedimented bacilli were washed in sterile distilled water, recentrifuged, and the resultant deposit suspended in 5 cc. of distilled water, to which 1 per cent of sodium carbonate had been added. The mixture was then heated for 30 minutes at 56°C. and kept for 24 hours at 37°C., so as to favor autolysis. Prior to injection, the autolysate was examined by means of Gram's stain and numerous shadow forms of the bacilli were seen. Cultivation tests demonstrated that the bacilli were dead.

A rabbit injected with 0.1 cc. of the autolysate of Shiga bacilli showed 4 days after inoculation a transitory mucous diarrhea of 2 days duration from which it promptly recovered. There were no signs of nervous involvement. A rabbit inoculated with 0.5 cc. of the autolysate presented paralysis of the anterior extremities after 24 hours and died after 48 hours. Autopsy revealed the typical nervous

[6] Only typical protocols are presented of a number of repeated experiments

lesions, together with a glazed appearance of the intestines, which were edematous and congested, and the intestinal contents gave a positive benzidine test for blood. Histologically, the appearances corresponded to those already described by Flexner and Sweet,[7] Doerr,[5] Dopter,[8] and others,[1] as a result of the action of Shiga bacillus toxin.

This experiment was repeated, employing, instead of a 3 day growth of Shiga bacilli in Noguchi flasks, a 24 hour growth. The results were practically identical; the dosage of the bacteria-free filtrate required to induce nervous lesions without intestinal involvement and the dosage of the autolysate of the bacilli necessary to produce intestinal lesions combined with nervous lesions were the same as in the case of the 3 day growth.

From the above experiment it will be noted that Shiga bacilli, Strain 109, when grown in a well aerated medium, forms in 1 to 3 days a soluble toxin which is recoverable in bacteria-free filtrates. This exotoxin exerts a typical action on the central nervous organs of the rabbit, producing mainly hemorrhages and necroses in the medulla and upper cervical cord, but not affecting the intestinal tract. On the other hand, autolysates of the bacterial bodies induce not only similar nervous lesions but also intestinal involvement. Therefore exotoxin, or neurotoxin, a product of the growth of Shiga bacilli, can be obtained in a pure state by this method; but endotoxin, or enterotoxin, the result of bacterial disintegration, is always admixed with a certain amount of exotoxin.

Experiments with Anaerobic Cultures.

The next experiments concerned the complete suppression of the neurotoxin-producing activity of Shiga bacilli by employing anaerobic methods and a study of the disintegration products of the bacterial bodies in such cultures.

Experiment 2.—For producing anaerobic conditions either of two methods, both giving practically the same results, was employed. The first method consisted in the inoculation of a tall column (15 cm.) of 1 per cent dextrose broth, pH 7.4, in a Noguchi test-tube, with a loopful of Shiga bacilli, Strain 109, grown pre-

[7] Flexner, S., and Sweet, J. E., *J. Exp. Med.*, 1906, viii, 514.
[8] Dopter, M. C., Les dysenteries, Paris, 1909, 75 ff.

viously for 18 hours in Brown's anaerobic jar.[9] The inoculated tubes were then placed in a Brown jar and incubated for 24 hours at 37°C. The growth of this tube was then subplanted into another and kept under similar anaerobic conditions. In this way the Shiga bacilli were subplanted to four consecutive anaerobic cultures, each incubating for 24 hours at 37°C. The fourth subplant was employed in the following experiment. The other method for obtaining anaerobic conditions consisted of layering over a similar column of the broth with petrolatum, then placing in an Arnold sterilizer and heating for ½ hour at 100°C. The medium was now anaerobic and was inoculated through the seal with 0.1 cc. of a Shiga bacillus broth culture grown previously for 18 hours in a Brown anaerobic jar. The inoculated, sealed tubes were kept for 24 hours at 37°C. With these exceptions, the method corresponded exactly with the one in which the Brown jar replaced the petrolatum seal for maintaining anaerobic conditions.

These anaerobic cultures were centrifuged at high speed until the supernatant fluid was clear. The latter was decanted, filtered through Berkefeld V or N candles, and the bacteria-free filtrate inoculated into the auricular vein of rabbits.

Rabbits which received 0.5, 1, 2, and 4 cc. of this anaerobic bacteria-free filtrate were unaffected and remained free from nervous or intestinal symptoms for a period of observation lasting 2 weeks or more.

The centrifuged, sedimented bacilli of these anaerobic cultures were, at the same time, washed in sterile distilled water, recentrifuged, and the clear supernatant fluid, or washings, decanted. The solid sediment of bacilli was suspended in 5 cc. of distilled water to which 1 per cent of sodium carbonate had been added. The mixture was heated for 30 minutes at 56°C. so as to kill the microorganisms, and then kept for 24 hours at 37°C. to favor autolysis. Prior to injection the autolysate was stained by Gram's method and cultured, and the mixture was found to consist of either completely autolyzed or dead bacilli. This material, in doses of 0.1, 0.2, 0.25, and 0.5 cc., was injected into the auricular vein of rabbits. The animals showed pronounced intestinal symptoms from 24 to 48 hours after inoculation, depending on the dosage. The first sign to be noted was straining and tenesmus, which was continual, accompanied by frequent evacuations of small, semisolid masses of feces. As the condition progressed, there was a mucous diarrhea streaked with either visible or microscopic blood, determined by the benzidine test. This condition persisted for 2 to 5 days, the animals returning, thereafter, to normal. Indeed, none of this series died. Some of the animals were killed at the height of the reaction for macroscopic and histopathological examination. The intestines then revealed grossly the typical glazing of the peritoneal coat, the edema, the injection of blood vessels, and hemorrhages, and showed microscopically the edema, hemorrhages, necroses, ulceration, and fibrinous (diphtheritic) covering of the epithelial coat, corresponding to the description given by Flexner and Sweet,[7] Doerr,[5]

[9] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677; 1922, xxxv, 467.

and Dopter[8] of the action of the Shiga poison, and later by Olitsky and Kligler[1] of the effects of Shiga endotoxin. In none of these animals were noted any signs of nervous involvement, either clinically or in section, even when ten times (1 cc.) of the minimal enterotoxic dose was used.

This experiment, typical of four similar ones, demonstrates that the exotoxic function of Shiga bacilli can be suppressed by growing the microorganisms under anaerobic conditions, either in fluid medium under a petrolatum seal or in this medium placed in an anaerobic jar. The bacteria, now in an exotoxin-free environment, can be autolyzed, or dissolved, with the resultant liberation of intracellular components, or endotoxin. The endotoxin, the preparation of which does not differ essentially from that of endotoxins from other bacteria, has a specific affinity for the intestinal tract, producing edema, hemorrhages, necroses, and ulcerations. The central nervous organs are not affected; hence the endotoxin can be properly designated as an enterotoxin.

We have thus separated the two toxins of the strain of *Bacillus dysenteriæ* Shiga which has been employed and have shown their biological distinctions. We now proposed to extend the observations of Olitsky and Kligler[1] by studying additional physical differences.

Diffusion of Toxins through Permeable Membranes.

The diffusion of the toxins through the walls of collodion sacs was investigated.

Methods.—Permeable collodion sacs for intraabdominal implantation and those for bacterial cultivation *in vitro* were prepared, following closely Gates' method.[10,11] For intraperitoneal implantation, the sacs were made on No. 12 size veterinary capsules;[10] for cultivation *in vitro*, the sacs were prepared in flasks.[12]

Preliminary experiments resulted in the choice of 2 per cent peptone broth (pH 7.4, for the reasons stated above) instead of the egg albumin broth which was suggested by Olitsky and Kligler[1] as the most effective medium. While the contents of the sac containing cultures of Shiga bacilli in the albumin broth were highly

[10] Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.
[11] Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 635.
[12] Gates,[11] p. 640.

toxic for rabbits, dialysates contained less toxin than that obtained by the use of a clear simple medium such as the peptone broth. The dense cultures of the bacilli in albumin broth coat the inner surface of the sac, thus preventing active dialysis.

Experiments with Sacs in Vivo.—Five rabbits were employed and into the abdominal cavity of each were placed two sealed sacs containing the broth inoculated with two loopfuls of agar slant cultures of Shiga bacilli. Four of the rabbits were unaffected for a period of 1 month. Then the sacs of two of the animals were removed and the contents were examined. A growth of Shiga bacilli was obtained on transplanting to agar, although a number were found autolyzed in stained preparations of the material. The contents of the sacs from one rabbit were reinjected into the auricular vein of two normal animals in doses of 0.1 and 0.05 cc. These latter died in 48 hours with typical nervous and intestinal symptoms. One of the rabbits containing the sacs was also injected at the same time with 0.1 cc. of the sac contents. This animal was unaffected. The experiment was repeated with similar results.

One of the four rabbits unaffected by harboring the sac cultures showed, 1 month after implantation, an agglutination serum titer of 1:1,280 against Shiga bacilli. The remaining three were negative.

The fifth rabbit exhibited, 5 days after implantation of the sacs, paralysis of both anterior extremities, paresis of the posterior extremities, and hyperesthesia. There were no intestinal symptoms. The animal died the next day, showing complete paralysis but no intestinal symptoms. The histopathology revealed the typical nervous lesions but the intestinal tract was normal. That these effects were not due to breakage of the sac and liberation of the bacteria was proved by culturing the sealed sacs after removal from the body for 48 hours in dextrose broth. No growth of Shiga bacilli occurred in the surrounding medium.

From these experiments it may be concluded that the Shiga bacilli, when grown in fluid medium contained in a sac which is implanted intraabdominally, survive and multiply therein for at least 1 month. During this period different effects are produced, depending, we believe, on the state of permeability of the sac. Rabbits harboring highly permeable sacs show the results of dialysis of pure exotoxin within 5 days after implantation; that is, the animal succumbs to lesions produced in the medulla and upper cervical cord. One may assume that if the exotoxin would dialyze out of the sac in nonlethal amounts the rabbit might exhibit at a later time intestinal symptoms due to subsequent disintegration of the bacilli within the sac with formation of endotoxin. Rabbits containing sacs of a lesser degree of permeability, although sufficient to allow an active diffusion

of the body fluids through their walls, produce a high titer of agglutinins in their blood sera. In this respect we have been able to confirm a previous observation of Gates,[13] who found that typhoid bacilli survive and multiply in sacs *in abdomine* for 1½ years; during this time agglutinins for these bacilli are produced. Even when agglutinins are not detectable in the serum of these animals, they remain immune to a later injection of either exotoxin or endotoxin.

The difficulty of determining the fine degrees of permeability of the sacs necessary to induce these effects renders this method unsatisfactory for our purposes of separating the two dysenteric toxins. However, certain conclusions may be drawn from these experiments: The contents of the sacs after 1 month *in abdomine* are highly toxic for normal rabbits, which show the effects of both exotoxin and endotoxin, corresponding to the multiplication and autolysis and dissolution proceeding in the sacs. Exotoxin diffuses through, primarily causing the death of the animal by the typical nervous lesions. Agglutinins are produced in surviving animals, in one instance in a titer of 1:1,280. Finally, immunity is conferred in the latter to subsequent injections of exotoxin and endotoxin.

Experiment with Sacs in Vitro.—For the purposes of these experiments, Gates' method of preparing sacs in flasks[12] was employed. 30 cc. of the same medium employed in the intraabdominal sacs were placed within the sac and 30 cc. of distilled water without. The medium within the collodion membrane was inoculated with two loopfuls of Shiga bacilli, Strain 109, grown for 18 to 24 hours on agar slants. The cultures were then kept for varying periods of time (from 3 to 8 days) at 37°C.

The cultures which were incubated 3, 4, and 5 days produced in the dialysate the typical exotoxin which induced in normal rabbits neurotoxic symptoms but no intestinal involvement. A single protocol is presented to show the manner in which an experiment was conducted and the results obtained. The protocol relates to one of a series of experiments made with a 5 day culture. The cultures kept at 37°C. for 3 and 4 days gave practically the same results, except that the nervous symptoms were of a milder degree and more transitory, enduring for 2 to 3 days, the animal returning thereafter to normal.

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[13] Gates, F. L., personal communication.

Experiment 3. 5 Day Culture.—

Rabbit.	Material injected intravenously.	Dose.	Symptoms and course following injection.
		cc.	
A	Dialysate.	10	48 hrs., paresis of right anterior extremity. 72 hrs., paralysis of both anterior extremities, hyperesthesia; paresis of posterior extremities. 4 days, condition unchanged. 5 days, condition improved; partial paralysis of anterior extremities; paresis of posterior extremities. 10 days, practically recovered. No intestinal symptoms during the 10 days.
B	"	10	48 hrs., hyperesthesia, paresis of posterior extremities. 4 days, paresis of anterior and posterior extremities. 7 days, partial paralysis of right anterior extremity; other limbs recovered. 11 days, practically recovered. No intestinal symptoms during the 11 days.
C	" + polyvalent antidysenteric serum* (injected simultaneously).	10 5	No effect.
D	Dialysate + polyvalent antidysenteric serum* (injected simultaneously).	10 5	" "
E	Dialysate heated at 75°C. for 1 hr.	10	" "
F† (immune).	Dialysate injected into rabbit which recovered from exotoxic paralysis 3 wks. previously.	10	" "

Rabbit.	Material injected intravenously.	Dose.	Symptoms and course following injection.
		cc.	
G	Contents of sac.‡	2	24 hrs., paresis of right anterior extremity; tenesmus. 48 hrs., paralysis of anterior extremities; paresis of posterior extremities; mucous diarrhea. 72 hrs., complete paralysis; diarrhea. Moribund; chloroformed. Nervous and intestinal lesions typical.

* This serum was the only therapeutic serum available. It was the Rockefeller Institute serum and contained exotoxic, endotoxic, and bacterial antibodies.[1]

† In another series of similar experiments with 5 day cultures, four animals which had been injected 2 to 3 weeks previously with exotoxin and had recovered from its action were reinjected with dialysate. They were all unaffected.

‡ On microscopic examination, the sac contents showed evidence of bacillary disintegration.

These experiments, which were repeated with similar results, demonstrate that the exotoxin is the first to appear in the bacteria-free dialysate. The exotoxin was identified by its power to induce nervous lesions, by its relative thermolability,[1] and by its failure to act in animals previously recovered from exotoxic effects (immunity tests), or when the toxin was injected simultaneously with immune serum. In the animals injected with the dialysates, no intestinal signs were observed, although on the 5th day the contents of the sac showed beginning autolysis, or disintegration, of the Shiga bacilli, and, consequently, caused both nervous and intestinal symptoms when injected into rabbits. However, after this period, and corresponding with an increasing degree of dissolution of the bacilli within the collodion membrane, both exotoxin and endotoxin appeared in the dialysates, as the following protocol shows.

Experiment 4. 6 to 8 Day Cultures.—Cultures such as were employed in Experiment 3 were allowed to incubate for 6, 7, and 8 days at 37°C. Normal rabbits were then injected with the different components of the cultures, as represented in the following protocol illustrating an experiment with a 6 day culture.

Rabbit.	Material injected intravenously.	Dose.	Symptoms and course following injection.
		cc.	
A	Dialysate.	10	24 hrs., paresis; mucous diarrhea. 48 hrs., paralysis and diarrhea. 72 hrs., completely paralyzed. Killed. Lesions in nervous and intestinal organs typical.
B	"	5	24 hrs., paresis of left anterior extremity and posterior extremity. 48 hrs., condition unchanged for 7 days thereafter. During the 9 days, no intestinal symptoms observed.
C	" heated for 1 hr. at 75°C.	10	Showed no nervous symptoms for 9 days; mucous diarrhea 3 days after injection which lasted 3 days; animal recovered thereafter.
D	Sac contents (containing numerous shadow forms along with viable Shiga bacilli).	1	24 hrs., paresis; mucous diarrhea. 48 hrs., paralysis of anterior extremities; paresis of posterior extremities; tenesmus; mucous diarrhea. Moribund; killed. Lesions in medulla, upper cervical cord, and intestines typical.

Experiments with 7 and 8 day cultures gave similar results, except that it required only 5 cc. of the dialysate to induce intestinal symptoms.

It appears from Experiment 4, therefore, that *in vitro* cultures of Shiga bacilli in collodion sacs produce mixtures of exotoxin and endotoxin in the bacteria-free dialysates. Hence, the early growths, that is up to 5 days incubation, yield pure exotoxin (Experiment 3) which diffuses readily through the collodion membrane and is detectable in the dialysate. After this period not only exotoxin but endotoxin diffuses through and the rate of the latter is proportional to the amount of dissolution, or disintegration, of the Shiga bacilli within the membrane. From the foregoing experiment it will be noted, as well, that the thermostable endotoxin in the dialysate can be separated from the relatively thermolabile exotoxin by heat.[1]

DISCUSSION.

Additional methods are presented to support the observations of Olitsky and Kligler[1] concerning the dual nature of the poison of Shiga bacilli. The first method consists of the suppression of the exotoxic, or neurotoxic, function of the microorganisms by anaerobiosis so that the disintegration of the bacteria, with consequent liberation of intracellular components, yields pure endotoxin, or enterotoxin. Thus the difficulty of securing pure endotoxin directly from the culture medium is overcome, for hitherto this toxin was always found admixed in the medium with exotoxin, which is an early product of the growth of Shiga bacilli.

The second method relates to the diffusion of these toxins through the walls of collodion sacs implanted intraabdominally or placed in flasks. Cultures in sacs implanted intraabdominally show different effects, depending on the permeability of the collodion membrane; highly permeable sacs allow the early passage of exotoxin, somewhat denser membranes permit only the diffusion of substances inducing agglutinins or immunity. Cultures in sacs placed in flasks yield in the fluid surrounding the membrane first exotoxin and later endotoxin. The passage through the membrane of endotoxin depends on the rate of bacterial disintegration within the sac.

Furthermore, the biological and physical differences of the two toxins which have already been demonstrated[1] are confirmed.

A practical bearing of this work concerns the preparation of an effective Shiga antidysenteric serum. The experimental production of antiexotoxin has already been described.[1] A potent serum for general use, however, should be anti-infectious and should contain endotoxic and exotoxic antibodies. The injection intravenously in horses of live Shiga bacilli, after the manner described by Flexner and Amoss,[14] has yielded a serum which contains 2,000 antiexotoxic units per cc., as well as endotoxic and bacterial antibodies (0.01 cc. of the serum neutralizes four minimal endotoxic doses and shows agglutinins ranging from 1:2,000 to 1:20,000 against Shiga bacilli[1]). Its anti-infectious potency as measured by Flexner and Amoss is such that 0.008 cc. protects rabbits against two lethal doses of Shiga bacilli.

[14] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 515.

CONCLUSIONS.

By the suppression, through anaerobiosis, of the exotoxin-producing activity of *Bacillus dysenteriæ* Shiga a pure endotoxin is produced directly from the culture. The duality of the poison of Shiga bacillus is further substantiated by studies on the diffusion of exotoxin, or neurotoxin, and endotoxin, or enterotoxin, by means of collodion sacs, implanted intraabdominally in rabbits or placed *in vitro*.

THE VIRULENCE OF AN EPIDEMIC STRAIN OF BACILLUS PESTIS CAVIÆ.

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(Received for publication, January 19, 1923.)

Certain experiments described in an earlier paper[1] indicated that the virulence of a given strain of mouse typhoid bacilli[2] for mice bred at The Rockefeller Institute is relatively constant. But although this work was done according to the usual bacteriological technique, it was felt at the time that any cultivation on artificial media between animal passages introduced a factor which might affect the infecting power of the various cultures. Consequently, the following experiments were devised to eliminate cultivation on artificial media and to determine the effect of animal passage on the virulence of a native pathogenic strain.

Experiment 1.—This experiment was planned to determine the effect of direct intraperitoneal mouse passage, without intermediate cultivation in artificial media, on the virulence of this strain of mouse typhoid bacilli.

Technique.—An 18 hour broth culture from the current stock strain was diluted 1:1,000 and injected intraperitoneally into ten mice weighing 16 to 18 gm. each. 24 to 48 hours later, when the animals began to show morbid symptoms, blood cultures were taken from the tail vessels. On the following day, the mice with positive blood cultures were anesthetized and bled from the heart. About 0.5 cc. of blood per mouse thus obtained was collected in salt solution containing 2 per cent sodium citrate. This suspension was immediately injected intraperitoneally into another series of ten mice. Coincident with the seventh direct passage in this manner, 1 cc. of the suspension was diluted and plated in the usual way to estimate the number of bacteria per cubic centimeter. Also control intraperitoneal titrations were run with the current stock strain used in the first passage series and with the original stock strain which had had no animal passage for over 3 years.

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[1] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.

[2] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 97.

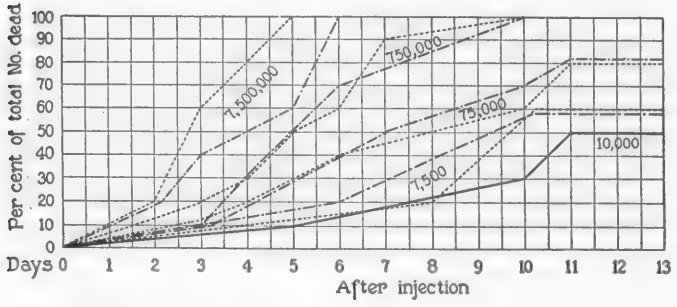
Table I and Text-fig. 1 compare the virulence of the three cultures. The mortality curves of the original culture and the current culture are quite similar for each dilution; the mortality curve of the "seven passage" mice, each of which received about 10,000 bacteria, compares with the curves of the control mice which received 10,000 and

TABLE I.

Duration of Life of Experimental and Control Mice Used in the Intraperitoneal Seventh Passage Test.

No. of mice.	Culture.	No. of bacteria injected.	Duration of life after injection.
			days
5	Original.	5,000,000	2, 3, 3, 4, 5
10	"	500,000	3, 3, 4, 5, 5, 6, 7, 7, 7, 10
10	"	50,000	3, 4, 5, 6, 8, 10, 11, 11*
5	"	5,000	8, 10, 10*
5	Current.	10,000,000	2, 3, 5, 6, 6
10	"	1,000,000	3, 4, 4, 5, 5, 6, 6, 7, 10, 10
10	"	100,000	4, 5, 7, 7, 7, 10, 10, 11*
5	"	10,000	6, 8, 10*
10	"Seven passage."	10,000	5, 10, 10, 11, 11*

* This test was terminated on the 13th day.



..... Original stock culture.
—· —· Current stock culture.
——— Current stock culture plus seven direct mouse passages.

TEXT-FIG. 1. The virulence of a strain of mouse typhoid bacilli after seven direct intraperitoneal mouse passages. To simplify the graph, the average number of bacteria per mouse in the two control series is given.

5,000 bacteria each, respectively. The test shows, then, that the virulence of this "seven passage" culture remains the same as that of the current stock culture and the original culture.

In order to continue the passage, this test was terminated on the 13th day. With the ninth direct passage, however, the series was allowed to run for 30 days, and a parallel control titration was employed again, using the current stock culture.

Table II and Text-fig. 2 show that the mortality rate of the "nine passage" mice, each of which received 1,000,000 bacteria, is about the same as the mortality rate of the control mice which received a similar number of bacteria of the current culture. It may be concluded,

TABLE II.

Duration of Life of Experimental and Control Mice Used in the Ninth Passage Test.

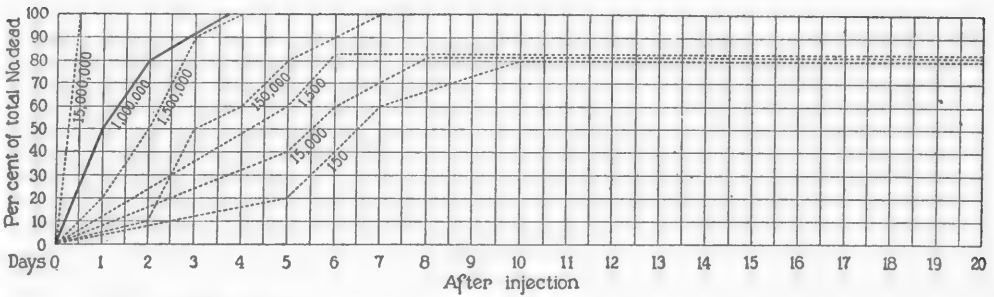
No. of mice.	Culture.	No. of bacteria injected.	Duration of life after injection.
			days
5	Current.	15,000,000	1, 1, 1, 1, 1
10	"	1,500,000	1, 1, 2, 2, 2, 3, 3, 3, 3, 4
10	"	150,000	2, 3, 3, 3, 3, 4, 5, 5, 6, 7
5	"	15,000	5, 5, 6, 8, S.*
5	"	1,500	5, 5, 5, 6, S.*
5	"	150	5, 6, 7, 10, S.*
10	"Nine passage."	1,000,000	1, 1, 1, 1, 1, 2, 2, 2, 3, 4

* Survived 30 days.

therefore, that nine direct intraperitoneal mouse passages with no intermediate cultivation on artificial media do not affect the virulence of this strain of mouse typhoid bacilli.

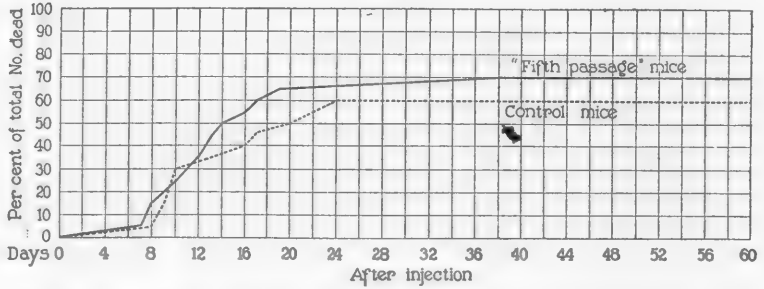
Experiments 2 and 3 were planned to determine the effect of direct *per os* mouse passages, without intermediate cultivation in artificial media, on the virulence of this strain of mouse typhoid bacilli. In Experiment 2, the bacterial suspensions were obtained from the heart's blood and spleen of animals in the acute stages of infection; in Experiment 3, the material was obtained from the intestinal contents of similar animals.

Experiment 2.—100 mice from the breeding room were given *per os* by stomach tube an 18 hour broth culture from the current stock strain, diluted 1:200. 9



.....Current stock culture.
————Current stock culture plus nine direct mouse passages.

TEXT-FIG. 2. The virulence of a strain of mouse typhoid bacilli after nine direct intraperitoneal mouse passages.



TEXT-FIG. 3. The virulence of a strain of mouse typhoid bacilli after five direct *per os* mouse passages (Experiment 2).

days later, 5 mice had died and 30 of the survivors showed positive blood cultures. These 30 mice were anesthetized, and for Experiment 2, 0.5 cc. of heart's blood from each mouse was put into salt solution containing 2 per cent citrate, and the spleen and heart were emulsified in 0.8 per cent salt solution in a sterile mortar. The two emulsions were combined. After slow centrifugation, the supernatant fluid was injected *per os* into the second series of 30 mice, each animal receiving 0.5 cc. of the suspension containing approximately 50,000,000 bacilli. This and all subsequent series were kept in 8 by 10 inch battery jars, 2 mice per jar. 11 days after the second series of 30 mice was injected *per os* with the blood and spleen suspension, 6 mice had died and 15 of the survivors showed positive blood cultures. From these 15 mice, heart's blood, heart, and spleen were taken, treated, and injected *per os*, as described above, into the third series of 30 mice. The number of bacilli given was this time about 5,000,000. 9 days later 6 mice had died. In the same manner, a blood and spleen emulsion was prepared from the survivors showing positive blood cultures and given *per os* to the fourth series of 30 mice of which each received about 25,000,000 bacilli. 14 days later, 7 of these mice were dead and 12 showed positive blood cultures. A blood and spleen emulsion was prepared as before from the 12 mice and injected *per os* into the fifth series of 20 mice, each receiving about 6,500,000 bacilli. As controls, 19 mice were given a 24 hour broth culture from the stock agar tube diluted so that each mouse received approximately 2,000,000 bacilli.

TABLE III.

Duration of Life of Experimental and Control Mice Used in the per Os Blood and Spleen Experiment (Experiment 2).

Culture.	No. of bacteria injected.	No. of mice.	Duration of life after injection.
			days
"Fifth passage."	6,500,000	20	7, 8, 8, 9, 10, 12, 12, 13, 13, 14, 16, 17, 19
Control.	2,000,000	19	8, 9, 9, 10, 10, 10, 13, 16, 17, 20, 22, 24

Table III and Text-fig. 3 compare the duration of life of the series receiving the mouse passage culture with that of the series receiving the unpassed stock culture. The mortality curves are so nearly identical as to indicate that the "fifth passage" culture possesses about the same degree of virulence as does the control unpassed culture.

Experiment 3.—Of the first 100 mice used in Experiment 2, 30 showed positive blood cultures on the 9th day. The small and large intestines of these mice were emulsified in salt solution, centrifuged, and injected *per os* into a series of 30 mice, each animal receiving 0.5 cc. of the suspension. 11 days after this second series of 30 mice was injected, 6 were dead and 15 of the survivors showed positive blood cultures. From these 15 mice, intestine and stool suspensions were made as described above, centrifuged, and injected *per os*, 0.5 cc. per mouse, into the third series of 30 mice. 9 days later, 7 mice had died and 7 showed positive blood cultures. The above technique was repeated and the resulting stool suspension injected *per os* into the fourth series of 30 mice. 13 days later, 5 of these mice had died and 10 showed positive blood cultures. The intestines from these 10 survivors were ground with sand, emulsified, and injected in 0.5 cc. quantities into the fifth series of 20 mice. The controls employed in Experiment 2 were used for this series.

TABLE IV.

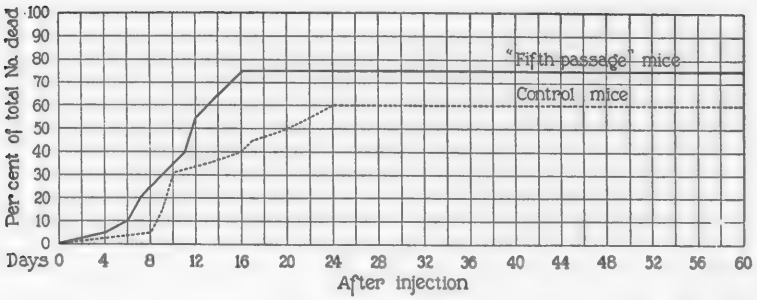
Duration of Life of Experimental and Control Mice Used in the per Os Stool Emulsion Experiment (Experiment 3).

Culture.	No. of bacteria injected.	No. of mice.	Duration of life after injection.
			days
"Fifth passage."	50,000,000*	20	4, 6, 7, 7, 8, 9, 11, 11, 12, 12, 12, 13, 14, 16, 16
Control.	2,000,000	19	8, 9, 9, 10, 10, 10, 13, 16, 17, 20, 22, 24

* This is merely a rough estimate based on spreading a measured amount of diluted stool suspension over green dye plates and counting the suspicious colonies after 24 hours incubation.

Table IV and Text-fig. 4 compare the duration of life of the series receiving the mouse passage culture with that of the series receiving the unpassed stock culture. The mortality curve of the mice receiving the stool suspension rises abruptly, in a manner similar to that observed when mice were sensitized with ox bile,[3] but the total mortality is not materially different from that of control mice. A rough estimation of the number of colonies per cubic centimeter in the stool suspension, determined by means of green dye plates, gave 50,000,000 per mouse in contrast to 2,000,000 per mouse in the control.

[3] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 33.



TEXT-FIG. 4. The virulence of a strain of mouse typhoid bacilli after five direct *per os* mouse passages (Experiment 3).

DISCUSSION AND SUMMARY.

Ever since Pasteur's demonstration of the modifying effect of animal passage on the virulence of bacteria, this device has been regarded as of great importance in intensifying infective capacity. It is not often, however, that extensive parallel tests have been made of the power of a microorganism to produce infection in a species of animal which is its natural habitat and under conditions in which the normal as well as artificial portal of entry is employed, and the potency of the microorganism passed directly from host to host is closely contrasted with that of one merely artificially cultivated outside the body.

The experiments described in this paper were arranged to fulfill these conditions. The results show that with the particular strain of *Bacillus pestis caviæ* used, successive animal passages do not modify infective capacity, or virulence. This finding is in harmony with the observations previously made from which it was concluded that the epidemic curve of mouse typhoid infection is explicable solely on the basis of bacillary distribution and host susceptibility.[4]

[4] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 269.

THE PRODUCTION OF ANTIBODIES IN RABBITS BY A SIMPLIFIED INTRATRACHEAL METHOD.

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(Received for publication, January 27, 1923.)

During the last few years interest has been revived in antibody production by means of injections into the trachea. Besredka[1] called attention to the laryngo-tracheal method of administering sera to guinea pigs. Among other experiments two guinea pigs were vaccinated with killed cultures of the diphtheria bacillus. One was treated intratracheally, the other inoculated into the peritoneal cavity. 11 days after the last injection each received an intratracheal injection of living culture. The animal that had been vaccinated by the intratracheal route survived; the other died. Both animals had developed agglutinins for the diphtheria organism.

Pfenninger[2] undertook a more detailed study of antibody production after the administration of antigens through the trachea. He incised the skin overlying the trachea and injected through a needle inserted between the cartilaginous rings. The injections were made 7 days apart. Comparisons were made of the intravenous, the intraperitoneal, and the intratracheal methods. Pfenninger pointed out that the intratracheal method for antibody production is a little better on the whole than the intravenous route. He considered both superior to the peritoneal route. Many of his rabbits died after several intravascular inoculations, but all those treated by the tracheal and peritoneal methods survived.

D'Aunoy[3] used much the same procedure. He concludes that the intratracheal injection is comparable with intravenous inoculation and superior to intraperitoneal injection for the production of agglutinins, bacteriolysins, bactericidins, and precipitins. Hemolysins were formed more slowly by intratracheal administration but ultimately the serum obtained was equal to that produced in the intravenous series. Attention is directed to the relative safety of the intratracheal method, since none of the rabbits died during the experiments. Several deaths occurred in the intravenous series.

A simple method for the administration of liquids into the trachea seemed desirable, preferably one which would produce the minimum amount of injury and

[1] Besredka, A., *Ann. Inst. Pasteur*, 1920, xxxiv, 361.

[2] Pfenninger, W., *Ann. Inst. Pasteur*, 1921, xxxv, 237.

[3] D'Aunoy, R., *J. Infect. Dis.*, 1922, xxx, 347.

enable the operator to inject animals rapidly. Factors other than the introduction of antigen into the respiratory tract have not been taken into account when injections through the walls of the trachea have been made. Winternitz, Smith, and Robinson[4] point out that injection of material through the walls of the trachea affords an opportunity for infection of the submucosa and the peritracheal tissue. They observed histological evidences of infection and were able to trace the injected organisms through the submucosa of the trachea and larger bronchi to the hilus of the lung by way of the peribronchial and perivascular structures.

Snel[5] had previously shown by experiments with guinea pigs that the anthrax bacillus failed to attack the intact lung. When the vegetative forms or spores were introduced by means of a catheter passed through the larynx, infection failed to take place. In guinea pigs injected by means of a needle inserted through the walls of the trachea a locus of infection was produced and the animals developed a septicemia.

Kitt[6] also cites similar observations by Arloing, Cornevin, and Thomas in immunization against blackleg. They showed that following the introduction of virulent material into the veins or the tracheæ, calves resisted subsequent subcutaneous or intramuscular injections. Great care was employed to avoid leakage into the perivascular and peritracheal tissues since the virus would multiply in these structures with fatal results.

An apparatus by which one could introduce fluids into the lower respiratory tract without appreciable injury to the mucosa and underlying structures would imitate more nearly the natural condition. In addition, a method which would enable one to space the doses at frequent intervals would be of considerable advantage.

Woven or rubber catheters introduced through the glottis have been employed by many for intratracheal inoculation, but considerable skill is required in their use.

Method of Intratracheal Injection.

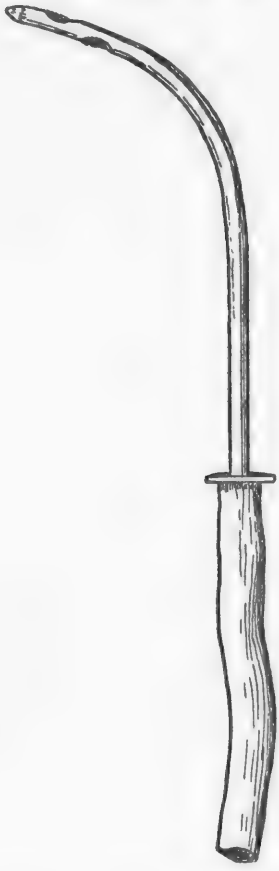
It has been stated that as simple a device as possible seemed advisable. It was found that a metal tube with a rounded end could be easily introduced into the trachea through the glottis. A milk or teat tube 9 cm. long, with an external diameter of 3 mm., was bent to a final angle of about 70° (Text-fig. 1). Such a tube has two open-

[4] Winternitz, M. C., Smith, G. H., and Robinson, E. S., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 195.

[5] Snel, J. J., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 103.

[6] Kitt, T., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1912, iv, 819.

ings on opposite sides situated a short distance from the end so that very little force is required to inject fluids. Rabbits are given sufficient ether to insure complete relaxation. The mouth is opened and the tongue grasped gently by means of rubber forceps and drawn



TEXT-FIG. 1.

TEXT-FIG. 2.

TEXT-FIG. 1. Tube for the administration of liquids into the trachea of rabbits. Actual size.

TEXT-FIG. 2. Tube for the administration of liquids into the trachea of guinea pigs. Actual size.

forward. The tube, previously immersed in paraffin oil, is inserted into the mouth and carried backward over the tongue and downward through the larynx. The tube is then connected to a syringe and the fluid slowly forced into the trachea. The tube is retained for a few seconds to allow drainage and then slowly withdrawn. After a little practise the whole operation may be done with considerable rapidity —usually from $1\frac{1}{2}$ to 3 minutes elapse between the beginning of anesthesia and the completion of the injection. The anesthesia may be so controlled that the animal has almost recovered when the tube is withdrawn.

A similar tube made from a $2\frac{1}{2}$ inch 14 gauge hypodermic needle will answer for guinea pigs (Text-fig. 2). Obviously the operation is more difficult in the guinea pig on account of the small mouth and the great development of the tongue at its base. A properly directed light is of considerable value when guinea pigs are used.

Comparison of the Production of Antibodies in Rabbits by Means of the Intratracheal and Intraperitoneal Routes.

Throughout the experiments the rabbits were immunized in pairs. The dose of antigen was always the same; the volume never exceeded 1.5 cc. In the case of bacterial and red cell suspensions the amount of material was increased from one series to another by concentration. Small volumes were indicated, since it was felt that the likelihood of all the material being retained in the respiratory tract was greater. All injections were made in series. Injections were made daily for 3 days, and 5 days after the last injection the animals were bled and the next series begun. The rabbits subjected to the peritoneal treatment were anesthetized for the same length of time as those injected into the trachea. Inasmuch as the protocols add little of interest, an outline of one is given in Table I.

In these experiments a heavy suspension in salt solution from a 24 hour agar culture of the hog-cholera bacillus was killed by heating to 60°C. for $\frac{1}{2}$ hour. This suspension was stored in the refrigerator as the stock vaccine. From it the antigens for the various series were prepared from week to week.

The results obtained in the case of these two rabbits have been recorded in Table II.

TABLE I.

Protocols of Rabbits Treated Intratracheally and Intraperitoneally with Killed Cultures of the Hog-Cholera Bacillus.

Rabbit No.	Method of administration.*	Series No.	Turbidity of antigen by Gates apparatus.	Time elapsing between beginning of anesthesia and completion of injection.	Thermic reaction.
				min.	
1	Intratracheal.	1	1.0	1½	None.
				2	Severe.
				1¼	"
2	Intraperitoneal.	1	1.0	1½	Slight.
				2	None.
				1½	"
1	Intratracheal.	2	0.8	1	Moderate.
				1½	"
				2	"
2	Intraperitoneal.	2	0.8	2	Slight.
				1½	"
				0	None.
1	Intratracheal.	3	0.5	2	Moderate.
				2	Severe.
				2	Moderate.
2	Intraperitoneal.	3	0.5	2	None.
				2	Slight.
				2	None.
1	Intratracheal.	4	0.4	2	Severe.
				2	Moderate.
				2	"
2	Intraperitoneal.	4	0.4	2	None.
				2	"
				2	"
1	Intratracheal.	5	0.3	2	Moderate.
				2	Severe.
				2	"
2	Intraperitoneal.	5	0.3	2	Slight.
				2	None.
				2	"

* The dose was always 1 cc.

TABLE II.

Agglutinin Production in Rabbits Immunized by the Intratracheal and Intraperitoneal Routes.

Rabbit No.	Method of administration.	Time.	Serum dilutions.													
			1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	1:10,000	1:20,000	1:50,000	1:100,000
		wks.														
1	Intratracheal.	1	C.*	C.	C.	++	+	±	±	−	−					
2	Intraperitoneal.	1	"	"	+++	+	+	+	±	−	−					
1	Intratracheal.	2	"	"	C.	C.	C.	C.	++++	++	+	±	−			
2	Intraperitoneal.	2	"	"	"	"	"	"	++++	+++	+	±	−			
1	Intratracheal.	3			"	"	"	"	C.	C.	++++	+++	++	+	−	−
2	Intraperitoneal.	3			"	"	"	"	"	"	+++	+++	++	+	±	−
1	Intratracheal.	4				"	"	"	"	"	++++	+++	++	++	+	±
2	Intraperitoneal.	4				"	"	"	"	"	C.	++++	+++	++	±	−
1	Intratracheal.	5					"	"	"	"	++++	+++	++	+	±	−
2	Intraperitoneal.	5					"	"	"	"	C.	++++	+++	++	+	±

* The readings were recorded as follows: C., complete clearing of the fluid; ++++, heavy deposit with slight turbidity of the liquid; +++, less marked agglutination; ++, moderate clumping; +, distinct deposit; and ±, slight deposit.

From the tables it will be noted that the temperature reactions following the intratracheal inoculation are on the whole more severe. The temperature of animals that have been vaccinated through the tracheal route usually falls 1°C. within an hour or two. This is followed by a rather sharp rise for several hours. The initial drop in temperature after the intraperitoneal administration is much less and the secondary increase is not so pronounced. After 3 or 4 weeks of treatment those animals injected intratracheally with bacterial vaccines usually develop a severe dyspnea immediately after the treatment. In two rabbits treated for considerable periods consolidation associated with *Bacillus lepisepticus* has been observed.

It will be noted that agglutinins developed to about the same degree in both animals. This has held true in experiments with other organisms.

Similar results have been obtained in the production of hemolysins and precipitins. For the production of a hemolytic serum rabbits were immunized with a suspension of the washed red cells of the sheep. As in the preceding experiments the amount of material injected at one time was limited to 1 cc. The animals received three series of weekly injections. The first series contained a 10 per cent suspension of red cells, the second a 20 per cent suspension, and in the third the cells were increased to 40 per cent. In this way constantly increasing doses of antigen were possible. The total number given was equivalent to 42 cc. of a 5 per cent suspension of sheep red cells. The results are given in Table III.

The rabbit treated by the intratracheal route formed hemolysin to a greater degree during the first series of inoculations than the other which received intraperitoneal injections. After the second series the animal injected intraperitoneally possessed the better serum. At the end of the third series the titer of both sera was about the same.

It was possible to produce a good precipitating serum in the same manner. In these experiments cow serum was administered by both routes. The maximum dose given at one time was 1.5 cc. A total of 12 cc. was administered over a period of 3 weeks. The results are recorded in Table IV. In more recent work it was found that 6 to 8 cc. of a foreign serum may be introduced into the trachea without causing noticeable ill effects.

TABLE III.

Hemolysin Production in Rabbits Immunized by the Intratracheal and Intraperitoneal Routes.

Rabbit No.	Method of administration.	Time.	Serum dilutions.										
			1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	1:10,000
		wks.											
3	Intratracheal.	1	C.*	C.	++++	+++	++	+	±	±			
4	Intraperitoneal.	1	"	++++	++	+	±	−	−	−			
3	Intratracheal.	2	"	C.	C.	C.	C.	C.	C.	C.	+++	+	−
4	Intraperitoneal.	2	"	"	"	"	"	"	++++	+++	±	±	−
3	Intratracheal.	3			"	"	"	"	C.	C.	++++	+	−
4	Intraperitoneal.	3			"	"	"	"	"	"	++++	++	±

* The usual method of recording hemolysis has been employed. The serum was stored in the refrigerator from 2 to 4 weeks before it was tested. Fresh guinea pig serum was used for complement.

During the first 2 weeks there was considerable difference in the antibody content of the two sera. At the end of the 1st week the serum of the rabbit (No. 5) immunized by intratracheal inoculation detected 1/1,000 cc. of cow serum; that of the other (No. 6) failed to react in the presence of 1/100 cc. By the end of the 2nd week the serum of Rabbit 5 had almost reached its maximum titer and reacted slightly with 1/20,000 cc. of cow serum. After 2 weeks 1/2,000 cc. of antigen was required to produce precipitation with the serum of Rabbit 6. At the end of the 3rd week both sera were equally efficient.

TABLE IV.

Precipitin Production in Rabbits Immunized with Beef Serum by Injection into the Tracheal and Peritoneal Cavities.

Rabbit No.	Method of administration.	Time.	Amount of antigen (in cc.) producing precipitation with 0.1 cc. of serum.*									
			1/100	1/200	1/500	1/1,000	1/2,000	1/5,000	1/10,000	1/20,000	1/50,000	1/100,000
		wks.										
5	Intratracheal.	1	+	+	+	+	±	−	−	−		
6	Intraperitoneal.	1	±	±	−	−	−	−	−	−		
5	Intratracheal.	2	+	+	+	+	+	+	+	±		
6	Intraperitoneal.	2	+	+	+	+	+	−	−	−		
5	Intratracheal.	3	+	+	+	+	+	+	+	+	±	−
6	Intraperitoneal.	3	+	+	+	+	+	+	+	+	−	−

* Definite precipitation has been recorded as +; ± indicates a slight turbidity and a trace of sediment.

DISCUSSION AND SUMMARY.

The method described of producing antibodies by the administration of antigens through the larynx is simple. The results obtained, however, conform closely to those obtained through intraperitoneal injection. The procedure is relatively a safe one and may well be employed in experimental inoculations. The advantages of rapidity and painlessness are obvious. In addition, gross injury has not been observed. Injections may be repeated at frequent intervals without danger to the life of the animal. The tube illustrated in Text-fig. 1

extends a little over 1 cm. into the trachea. The tube designed for the guinea pig reaches 2 or 3 mm. below the glottis. While the doses indicated in the protocols are small, 7 to 10 cc. of liquid have been given to rabbits by means of the tube without ill effects. It has been shown by the injection of India ink that the material is well distributed throughout the lungs in both the rabbit and guinea pig.

Under certain conditions it may be advisable to inject more deeply into the trachea, especially in the rabbit. The tube illustrated in Text-fig. 1 is not adaptable for this purpose. A cannula of larger diameter has proved of distinct advantage as a shield for introducing flexible catheters well down the trachea. A cannula 9 cm. long and 4 mm. in diameter when bent at an angle of 45° may be passed through the larynx of a rabbit without difficulty. A No. 8 (French) woven or No. 10 soft rubber catheter may be inserted into the cannula beyond the bend before the tube is passed through the glottis. After the tube has entered the glottis the catheter then may be introduced as deeply as desired into the teachea. The metal cannula enables the operator to "feel" the glottis.

From the experiments one seems justified in concluding that the results obtained by administering antigens by way of the trachea are about the same as those obtained by the intraperitoneal route. With the tubes described injury has been largely eliminated. The procedure is relatively a safe one.

STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

III. Intratesticular Transplantation and Clinical Course of the Disease.

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When a satisfactory method had been devised for the transplantation of the tumor which forms the subject of this series of papers (1), a systematic study of the tumor was undertaken, using the method of correlation of clinical and pathological phenomena. The immediate object of these investigations was to obtain a general conception of the behavior of the tumor as an agency for the production of disease, with a view to utilizing this knowledge as a basis for future work. It had been found (1) that under favorable conditions, the tumor was capable of producing a very maligant disease, while under other conditions, it was difficult to obtain even a primary growth. It appeared, therefore, that by the use of a variety of methods of transplantation, one might obtain results which would permit an estimation of the relative importance of the several groups of factors which were concerned in determining the results of transplantation and would give some insight into their mode of operation.

These investigations necessitated a long series of experiments which extended over a period of nearly 2 years. During this time, the tumor was passed through more than twenty generations; inoculations were made into the testicles, the skin and subcutaneous tissues, the muscles, the anterior chamber of the eye, the brain, the peritoneal cavity, and the blood stream. All these animals were studied clinically and pathologically as individual subjects of disease. Thus far, only male rabbits have been used; they have been carefully selected and have been kept in individual cages, and given the best care and attention. With this material, we have been able to study phe-

nomena of tumor growth and of animal resistance, and to correlate these with clinical and pathological effects under favorable and unfavorable conditions of transplantation.

In reporting the results of these investigations, it seems advisable to begin with a description of the growth obtained by various methods of transplantation and the clinical effects observed in tumor-bearing animals, in order to provide a general perspective of the disease to which individual phases of the subject may be related. The present paper will deal, therefore, with the results obtained by testicular inoculation, while the results of other methods will be presented in the next paper of the series (2). In view of the complexity of the conditions which influence the results of transplantation in one way or another, statistical data will be omitted as far as possible until such time as they can be introduced and analyzed to advantage. At present, any attempt to deal with the subject from this standpoint would only lead to confusion unless accompanied by a great deal of explanatory material which would carry us beyond the limits which have been assigned to these papers.

Intratesticular Transplantation.

Methods and Material.

The description of the results obtained by intratesticular transplantation is based upon the study of a large series of rabbits. These animals were inoculated with tissue fragments or with a cell emulsion prepared according to the method described in a previous paper (1). With few exceptions, they were inoculated in only one testicle, which was usually the right, so that there was only one primary focus of tumor growth.

Results.

Percentage of Tumor Growths.—Intratesticular inoculation of finely minced tumor tissue or of a homogeneous cell emulsion prepared from actively growing tumors yielded a definite growth in nearly all animals. Occasionally, the growth was slight, and, in rare instances, no growth could be recognized by ordinary methods of clinical examination, but the percentage of positive results was rarely less than 90 and as a rule was 100 per cent.

Incubation.—Inoculation was usually followed by a slight diffuse swelling of the testicle which disappeared within 24 to 48 hours, leaving a small area of thickening or induration at the point where the tissue was discharged. This in turn diminished or disappeared within 3 to 5 days and was followed immediately by the development of one or more circumscribed nodules or by a diffuse enlargement of the testicle with ill defined areas of thickening. These changes marked the appearance of a definite tumor growth. During the earlier generations, this period varied from 5 to 10 days, but with later generations, the time has been appreciably shortened and now rarely exceeds 5 days.

Growth Characteristics.—The growth of the tumor was usually periodic in character. As far as could be determined by palpation, there was a steady increase in size for a period of 2 to 3 weeks before any interruption occurred. By this time, the tumors in the majority of animals had reached a size of from 1 to 3 cm. in diameter. Occasionally, complete regression occurred after this first cycle of growth. This was especially true of slowly growing tumors in young or adult animals.

In a second group of animals with unusually active tumor growths, there was apt to be a sudden development of edema in the scrotum and adjacent tissues, followed by rapid regression, the tumor softening and diminishing in size. This phenomenon of crisis, as it may be termed, was frequently followed by complete resolution of the primary tumor, but many of these animals subsequently developed secondary growths. In other instances, the primary tumor was only temporarily affected.

The course of events in a third and comparatively large group of animals differed from that of the second in that the edema was less marked, and while growth was arrested, there was comparatively little regression, or even though there was an extensive edema, little or no regression took place.

Finally, there was a fourth group of animals in which growth of the primary tumor was active or of moderate degree but progressed with only slight or brief interruption at this period.

Small or feebly growing tumors, which formed only a small proportion of the whole, not infrequently began to regress at about the end

of the 2nd week, but usually the first critical period in the growth of primary tumors occurred at or near the end of the 3rd week after inoculation and was remarkably uniform in this respect. This was not peculiar to testicular tumors but was observed after all forms of inoculation and is worthy of especial emphasis as a feature of the behavior of the transplanted tumor.

The interruption of growth which occurred at this time was usually of short duration and rarely extended beyond the 4th week. Growth was then renewed with greater or less activity. In many instances, the primary tumor increased actively, involving the entire testicle and extending up the cord into the abdominal cavity in the form of a continuous mass or as discrete nodules which might be few in number and comparatively large, or small and very numerous. It sometimes happened that the testicle was drawn into the abdominal cavity and growth continued in this location. In other instances, a testicular mass developed which involved tunics and scrotum and not infrequently reached a size of 5 to 7 cm. in length by 2 to 3 cm. in diameter.[1]

In a smaller proportion of the animals, the growth which occurred after the interruption of the 3rd or 4th week was less rapid than before or was more circumscribed in character. In some instances, the primary tumor remained comparatively small (1 to 3 cm. in diameter), while in others, it reached a very large size but was confined within the scrotum.

After the 4th or 5th week, periodic changes in the activity of primary tumors were less evident. By this time, the animals of a series had become separated into three fairly distinct groups. The first included animals in which growth appeared to have reached its limits, and the tumors were either quiescent or definitely regressing; the second group was composed of animals with slowly but irregularly growing tumors; while the third was composed of animals with actively growing tumors. With few exceptions, however, what took place was still of an intermittent character, resolution being interrupted from time to time by periods of quiescence or even by a slight renewal of growth, while the growth of more active tumors was

[1] See Pearce and Brown (1), Figs. 1 to 4.

likewise interrupted by periods of quiescence or slight regression. The exceptions to this rule occurred with tumors of an unusually malignant character and less often in instances of unusually rapid resolution in which either process took place with no appreciable interruption.

Metastases.—Metastases developed in the majority of animals, and there were few organs or tissues in which secondary growths were not found, but a detailed report of this feature of the transplanted tumor will be reserved for a future communication. At present, it is sufficient to say that extension of the growth through the inguinal canal and the formation of implantation metastases in the abdominal cavity occurred as early as the 3rd week after inoculation, and metastases by lymph or blood vessels to distant parts of the body made their appearance anywhere from the 3rd to the 5th week.

Termination.—The ultimate fate of the tumor or of the tumor-bearing animal was variable and depended upon a number of factors to be discussed in a subsequent paper. As has been intimated, the growth was suppressed very quickly in some instances; while in others, resolution and apparently complete recovery took place after the tumor had grown for several weeks or months, and this sometimes occurred even after extensive metastases had developed. More often, however, the occurrence of metastases was eventually followed by death. In some instances, death was due to mechanical causes, such as intestinal obstruction, or pressure on the spinal cord, or to mechanical interference with nutrition as in the case of metastases affecting the jaws and especially the region of the lower incisors, but a considerable proportion of the animals showed none of these complications.

Finally, there was a third group of animals in which a condition of equilibrium became established, and for months there was no decided change in one direction or the other, leaving the outcome uncertain even after prolonged observation.

The probable mortality in different series of animals cannot be estimated with accuracy owing to the fact that few series were held intact for more than 2 to 3 months and that it is impracticable to prolong the period of observation sufficiently to obtain a definite end-result. In general, it may be said, however, that within a period of 2 to 3 months, the mortality varied from nil to 100 per cent, depending

upon a number of factors, such as the composition of the series as regards the age and breed of the animals used, the state of the inoculating material, and especially the season at which the experiments were carried out. Under ordinary conditions, from 20 to 30 per cent of the animals apparently recovered during an observation period of 2 to 3 months, while 20 to 40 per cent died, leaving from 40 to 60 per cent in which the outcome was not determined. These proportions are significant as indicating a resistance distribution which is relatively constant for different groups of rabbits. That is, in any group of five rabbits, there are usually one of relatively high resistance, one of distinctly low resistance, and three which are intermediate and comparable to one another.

Duration.—For the same reasons as those given above, the duration of tumor growth or the length of survival of tumor-bearing animals can be stated only in general terms. The growth of the tumor in surviving animals varied between 10 days to 2 weeks and an indefinite period of more than 6 months. Few animals showed complete suppression of tumor growth before the end of the 2nd or 3rd week. In most instances, final regression began between 6 weeks and 2 months after inoculation. That is to say, the turning point was reached at about this time, and one was able to forecast the probable outcome with a considerable degree of accuracy. Resolution and absorption required a longer time, and in the case of large tumors were rarely complete before the end of the 3rd month, and frequently the time was much longer.

Death from the tumor usually occurred between 7 and 12 weeks after inoculation, the period of greatest frequency being from 7 to 10 weeks. Occasionally, the course of the disease was more prolonged, and the animal survived 4 to 6 months after inoculation. More often, the disease was fulminating in character, and death occurred within 6 to 7 weeks.

Relation between Growth and Malignancy.—In concluding the section dealing with phenomena of tumor growth, it should be made clear that no constant relation was observed between the rate of growth or size of primary tumors and the malignancy of the tumor as indicated by the occurrence of metastases or by the course of the disease. In fact, it appeared that the relationship was more often a

reverse than a direct one. That is, tumors which grew most actively during the first 2 to 3 weeks were usually suppressed and rarely gave rise to extensive metastases or caused the death of the animal. In other instances, large primary tumors developed which were confined within the tunics and the scrotum and produced no serious consequences. The most malignant tumors, on the other hand, were characterized by a moderate but steady rate of growth with invasion of the tunics and extension up the cord into the abdominal cavity. Similar conditions obtained with reference to metastatic tumors. The most distinctive feature of the two classes of tumors was, therefore, the manner in which the animal responded to the presence of the tumor rather than the rate or size of the growth.

Immunity.—The subject of immunity of tumor animals was not investigated extensively, but it may be said that, with rare exceptions, animals that had once been inoculated with negative results were insusceptible to a second inoculation. In like manner, animals that had apparently recovered could not be reinoculated successfully by any of the usual routes. The duration of this resistance is not known, but reinoculations made as late as 6 months after recovery still gave negative results.

Symptomatology of Tumor Animals.

In describing the symptomatic effects produced by this tumor, we shall confine ourselves almost entirely to such conditions as can be detected by ordinary methods of observation supplemented by records of weight.

The first recognizable change which occurred in tumor animals was of a local character and consisted of an enlargement of the regional lymph nodes which appeared almost immediately after inoculation and gradually increased for upwards of 7 to 10 days. This reaction in the regional lymphatics was usually followed by similar changes in other groups of nodes. As a rule, the initial swelling diminished towards the end of the 2nd week, leaving the nodes still somewhat enlarged and distinctly indurated. This condition persisted with but little change until the turning point in the disease was reached. At that time, the nodes showed either a further increase in size or underwent a decided atrophy. Thus, if the growth was progressive,

further enlargement of the lymph nodes usually occurred, but when resolution took place, the nodes diminished in size and in some instances became extremely small and indurated. In this respect, there was a striking contrast between the conditions presented by animals in which the disease pursued a rapidly fatal course and those which survived for a longer time. In acutely fatal cases, the lymph nodes were usually smaller than normal, whereas in the more chronic cases, they tended to be greatly enlarged and were succulent or edematous in character. During the late stages of the disease, therefore, either a hypertrophy or atrophy of the superficial lymph nodes might occur, depending upon the course of the disease.

During the first few weeks after inoculation, there were no decided changes in the general physical condition of animals with actively growing tumors. They usually gained in weight, and at times, the increase was somewhat greater than that of normal animals under similar conditions, and was usually greater than that of animals in which the growth of the tumor was slight or of short duration. In fact, these animals not infrequently showed an actual loss in weight.

The first evidence of a nutritional disturbance appeared as an accompaniment of the phenomenon of suppression of tumor growth and consisted of a cessation of the normal increase in weight or a sudden loss in weight which in extreme cases amounted to several hundred grams. This condition might be transient or might last for from 10 days to 2 weeks, and was frequently associated with some change in disposition. In the milder cases, this usually took the form of a nervous excitation, while in other instances, there was a decided apathy with dryness of the skin, roughening of the coat, and loss of appetite which doubtless accounted for some part of the loss in weight and the prolongation of this condition.

These changes in physical condition appeared to be influenced largely by the extent of the tumor growth and by the suddenness with which the reversal of this process took place. With slight or transient growths, there might be no appreciable alteration in the physical condition of the animal. If, on the other hand, the growth had reached a considerable size and regression began abruptly, the change was usually marked. In some animals with large primary tumors or with metastases to internal organs, in which the change

from progression to regression took place slowly, the loss in weight might be barely perceptible, while the change in disposition was very decided. In some instances, there was no more than an apathy, while in others, there was a marked nervousness which was frequently accompanied by a pronounced exophthalmos. This latter condition was also noted in other classes of animals. It was frequently transient, but at times persisted for weeks, or disappeared and recurred from time to time with successive periods of progression and regression of the tumor. It was not permanent, however, but disappeared as the tumor was absorbed.

As absorption of the tumor progressed, the general condition of the animal tended to improve. Animals that had made a prompt recovery showed a decided improvement in their general appearance, with a substantial increase in weight, and remained in excellent physical condition for months. In cases in which recovery was slow and at times uncertain, there was evident impairment of physical condition and the animals were subject to intercurrent infections and their powers of resistance to such infections were diminished as contrasted with those of the first group.

When the growth was not arrested, the disease followed either an acute or chronic course, terminating in death, and the changes observed in the physical condition of the animal varied accordingly. Those animals in which the tumor grew most actively usually remained in excellent condition until shortly before death. If death occurred suddenly as a result of accidental interruption of some essential process, there might be no obvious deterioration in the physical condition of the animal, depending upon the progress which the disease had made at the time of such an occurrence. In the absence of accidental complications, the animal began to lose weight and strength, the conjunctivæ and mucous membranes became pale, movements were sluggish, the skin was relaxed and was thin and delicate, while the coat was dry and roughened, the eyes were sunken, the lids drooped, and towards the end, there was not infrequently an excessive flow of lacrimal and mucous secretions.

The pulse rate of these animals was so variable that no estimation of its character can be given. The temperature during the early periods of tumor growth varied very little from the normal, but as the

disease progressed, there was an apparent tendency to an elevation of temperature which was relatively slight in most animals. Occasionally, however, the temperature was subnormal, and a decided elevation followed by a fall below normal immediately preceding death was noted in several instances.

The conditions described made their appearance anywhere from a few days to 2 or 3 weeks before death and increased with greater or less rapidity according to the progress of the disease. It appeared that these symptoms were rarely if ever the result of tumor growth *per se*. In fulminating cases of malignancy, metastases were nearly always present in one or both suprarenals, and they were frequently found in the pituitary body, hence it is difficult to determine the part played by tumor growth and that which may be ascribed to accidental involvement of organs such as these.

In this connection, mention should be made of the occurrence of clinical conditions simulating Addison's disease—minus the element of pigmentation—and of genital atrophy from metastases in the suprarenals and pituitary body respectively, and the occasional occurrence of a peculiar skeletal overgrowth for which as yet no cause can be ascribed. The conditions which suggested the Addison syndrome occurred in their most typical form in animals which did not show extensive tumor metastases, the presence of metastases in the suprarenals being indicated by an extreme weakness and pallor of mucous membranes which were out of proportion to other evidences of tumor involvement. The picture was so characteristic as to lead one to believe that a close approximation of the human disease might be produced experimentally by inoculations made directly into the suprarenal glands.

In like manner, the presence of a metastasis in the pituitary body was diagnosed on several occasions by the occurrence of an extreme atrophy of the uninoculated testicle which under ordinary circumstances retained its normal size and condition or underwent an hypertrophy. The other element of the Fröhlich syndrome (adiposity) was rarely seen in these animals, due to the presence of metastases in other organs or to the acute course of the disease, but in one instance, the complete picture of marked accumulation of fat and extreme atrophy of the testicle occurred in typical form.

The picture presented by animals in which the disease pursued a chronic course extending over some 4 to 6 months was quite different from that of the acutely fatal affection. The change from a condition of well-being to one of extreme cachexia occurred insidiously, and at the end, the picture was complicated by the presence of some intercurrent infection. There was a gradual loss of weight and of strength; the animal became apathetic and the movements were sluggish; the hair was dry and roughened and the eyes sunken as in the preceding case, but the skin of these animals was also dry and thick, or of a parchment-like texture, and was covered by fine scales, and frequently there was a decided thinning out of the hair. In addition, areas of mucoid infiltration were frequently present in the skin and subcutaneous tissues of the genital region, and as the end approached, they occasionally appeared elsewhere. These animals maintained a firm hold upon life in the presence of extensive tumor metastases, and death was not infrequently due to some form of bacterial infection.

It is obvious that conditions other than those described were present in all classes of tumor-bearing animals, but as yet there are many aspects of the disease which we have not been able to investigate in a systematic way. The blood, for example, showed distinct changes, the best known features of which were an anemia, a mononucleosis, and some change in coagulability. Under certain circumstances, the coagulation time was diminished, but in cases of high malignancy, it was apt to be increased and instances were noted in which the blood either failed to coagulate after prolonged standing or formed a very imperfect clot.

CONCLUSION.

Since the paper which follows this will deal with the results obtained by other methods of inoculation, the discussion of the transplanted tumor and its effects upon the animal organism may be conveniently deferred until the report dealing with these subjects has been completed. From what has been said, it is apparent, however, that when transplanted by the method described, the tumor under consideration is capable of producing a highly malignant disease. But even under these conditions, the results of transplantation are

extremely variable, which indicates that the factor of animal resistance is of equal if not greater importance than that of the capacity for growth possessed by the tumor cells.

SUMMARY.

The results obtained by intratesticular inoculation of a malignant tumor of the rabbit are presented in the form of a general summary covering the phenomena of growth and spontaneous regression of the tumor and the clinical evidence of disease in tumor animals.

Under ordinary circumstances, growth is obtained by this method of inoculation in practically all animals, and the majority of them show metastases.

The ultimate fate of tumor animals is variable. Spontaneous regression is of frequent occurrence, and apparent recovery may take place even after extensive metastases have developed. More often, the disease terminates fatally. The course of the disease may be fulminating in character with death occurring 6 to 7 weeks after inoculation, or it may be prolonged over a period of more than 6 months, but death usually occurs between 7 weeks and 3 months after inoculation.

The symptomatology varies with the course of tumor growth but is in general that of a debilitating disease of an acute or chronic character in which the picture is frequently complicated by symptoms referable to mechanical causes or to one or more of the glands of internal secretion. The most prominent of these are due to the presence of metastases in the suprarenals or pituitary body, in which case symptoms of Addison's disease or diseases of the pituitary may be superimposed upon those attributable to the presence of extensive tumor growths. In the absence of such growths, however, the symptoms of suprarenal or pituitary involvement may dominate the picture.

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STUDIES BASED ON A MALIGNANT TUMOR OF THE RABBIT.

IV. The Results of Miscellaneous Methods of Transplantation, with a Discussion of Factors Influencing Transplantation in General.

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Plates 44 and 45.

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As stated in previous papers of this series (1, 2), rabbits inoculated in the testicles with tissue fragments or a cell emulsion of the tumor under consideration almost invariably develop a local growth. These tumors frequently metastasize and give rise to physical signs and symptoms of disease which differ somewhat according to the course of the disease and the location of metastases in individual animals. Under favorable conditions, the progress of the growth is rapid, and while some animals apparently recover, many of them die either acutely (6 to 8 weeks), or after the lapse of several months, presenting a picture not unlike that of malignant disease in man.

In reporting the results of intratesticular inoculation, emphasis was placed upon the phenomena of disease or the clinical effects of the tumor and the reaction of tumor-bearing animals rather than upon the immediate results of inoculation. When other methods of transplantation are employed, however, conditions are altered to such an extent that the results must be presented from a different point of view. The percentage of takes, the size attained by primary tumors, the incidence of metastases, and the duration of the growth become the standards of measurement as in most experimental work with the tumors of laboratory animals.

Thus far, the efforts which have been made to propagate the tumor may be divided into three classes: (1) inoculation of uninjured tumor

fragments or tumor cells; (2) inoculation of cells which have been injured, as by repeated freezing and thawing; and (3) inoculation of desiccated tumor tissue and of Berkefeld filtrates. A few attempts have also been made to transmit the growth to other species of animals. The results of experiments other than intratesticular inoculation of uninjured tumor cells will be reported in the order enumerated above.

Transplantation of Uninjured Tumor Cells.

In addition to the intratesticular route, seven methods of transplantation have been studied, including subcutaneous, intracutaneous, intramuscular, intraocular, intracerebral, intraperitoneal, and intravenous inoculations. For convenience, the results obtained by these methods may be considered collectively.

Methods and Material.

The selection and preparation of the material used for all inoculations in this group were essentially the same as those described in the second paper of the series (1) and need not be repeated. Both tissue fragments and cell emulsions have been used for subcutaneous and intracutaneous inoculations, but cell emulsions were used for all other inoculations. Injections were made with a syringe and needle (Fournier tuberculin syringe, graduated in 0.01 cc.).

Subcutaneous inoculations were made in the scrotum, the groin, and the axilla or pectoral region, intracutaneous inoculations on the ventral surface of the sheath, and intramuscular inoculations in the muscles of the inner and posterior aspect of the thigh.

Intraocular injections were carried out under a local anesthetic. The cornea of one eye was punctured at its upper and outer margin with a corneal knife; a small amount of the aqueous humor was permitted to escape and the emulsion introduced into the anterior chamber.

Intracerebral inoculations were done under a general anesthetic. A small incision was made in the skin over the middle of the right frontal bone near its anterior extremity. With a trephine, a small opening was then made in the bone through which the needle was introduced and the injection made into the anterior portion of the

right frontal lobe of the brain. These procedures were carried out aseptically.

The intravenous inoculations were made through an ear vein and the intraperitoneal by injection into the peritoneal cavity.

For the sake of uniformity, male rabbits were used in all cases, and with the exception of one group of experiments, in which combined intratesticular and subcutaneous or intracutaneous inoculations were studied, animals were inoculated at only one point.

In most instances, a single series of five or six animals appeared to be sufficient to indicate the results which might be expected from the use of a given method and the probable utility of the method for experimental purposes, but with the intracutaneous and subcutaneous routes, several series of animals were studied.

Results.

Tumor Incidence.—Inoculations made into the brain and the anterior chamber of the eye gave positive results in all but one animal. In this instance, the emulsion was apparently washed out of the eye and a second inoculation was successful. The percentage of takes from intramuscular and intracutaneous inoculations was also high (80 to 90 per cent), but with the subcutaneous route, it was often difficult or impossible to determine whether there was a definite growth or not on account of the marked local reaction. A small nodule developed at the site of inoculation in all animals, but in most instances, this nodule was composed largely of granulation tissue. From a study of these lesions, it appeared that the cells introduced survived for several days and that there was probably some growth but it was only in an occasional animal that a clearly defined tumor developed.

If for present purposes, therefore, such indefinite results be regarded as negative, the incidence of tumors from subcutaneous inoculations may be placed at from 20 to 25 per cent, or one animal out of four or five. For example, in a series of sixteen rabbits inoculated in the subcutaneous tissues of the groin, four developed fairly large, actively growing tumors, and in another series of fourteen animals, three developed tumors. Only once was this proportion exceeded.

In this instance, three out of five animals gave positive results, but there was still only one animal with a good tumor growth comparable to those regarded as positive in other series.

Attention may be called to the fact that this ratio of one in five corresponds roughly with the proportion of fulminating cases of malignancy which occurred after intratesticular inoculation and that it is also significant in that it corresponds with an incidence of low resistance displayed by rabbits to a variety of unrelated experimental conditions. It has been given practical application in this laboratory for a number of years in planning and evaluating toxicologic experiments and experiments dealing with biological aspects of infections such as syphilis. Since no normal rabbit has died thus far from tumor inoculations in the subcutaneous tissues, this method of inoculation has been utilized in the study of crossed relationships of resistance to tumor and to syphilis as one means of identifying animals with the lowest tumor resistance. The reactions to intracutaneous and intramuscular inoculations have also been helpful in this respect as a means of determining intermediate degrees of resistance.

Intraperitoneal and intravenous inoculations, as determined by autopsy, gave practically the same results as subcutaneous inoculation. Each of these series consisted of five rabbits. One animal of each group was killed at the end of 3 weeks; one of the intravenous animals became very weak and was killed after 4 weeks, while the others were killed at from 6 to 8 weeks after inoculation. The results of the two series were identical. In each, there were three animals with no evidence of active or healed tumors, one animal with what appeared to be small tumor nodules which had healed, and one with active multiple growths. Both of these were diagnosed during life, in one case by the development of weakness and anemia suggestive of Addison's disease (intravenous series) and in the other by a palpable growth in the abdominal wall.

Incubation.—The incubation period which might be given for tumors produced by different methods of inoculation would naturally vary according to the opportunity for detecting the presence of a growth. Thus, with the intravenous and intracerebral inoculations, the first evidence of a growth was symptomatic, and in most instances,

these symptoms did not appear until nearly 3 weeks after inoculation. With inoculations made into the anterior chamber of the eye on the other hand, a visible growth could be detected within 72 hours. In other instances, the first signs of growth were derived from palpation, and the results varied somewhat according to the extent of the reaction aroused in the tissues at the site of inoculation.

However, a growth in the skin or muscles was usually recognizable within 5 to 7 days after inoculation, and within 7 to 14 days after inoculations in the subcutaneous tissues.

Growth and Termination.—The character and extent of the growth obtained in these experiments differed according to the method used. It was found that during the first few weeks the growth of the tumor could be followed to excellent advantage in the anterior chamber of the eye, which served the purpose of a culture medium in which the proliferation of tumor cells and the growth of nutrient vessels could be observed directly. In this location, the growth first assumed the form of an extremely delicate membrane or of an intricate network of fine thread-like processes of a pale opalescent appearance which floated freely in the aqueous humor, except for an attachment at their point of origin. The growth of these structures was accompanied by the development of an equally delicate network of vessels which originated from subjacent parts of the iris. These vessels were so delicate that they were apt to be ruptured by any unusual excitement of the animal, and hence were the source of rather frequent and profuse hemorrhage.

For the first 2 or 3 weeks, the growth of these tumors was very rapid, and by the end of the 3rd week, the anterior chamber was usually filled with an irregular lobulated mass of an opaque white or opalescent appearance mottled with areas of necrosis and hemorrhage. When this point had been reached, growth became less active on account of the confining influence of the tunics of the eye. Eventually, however, the bulb became deformed and tended to protrude, and in some animals, the growth penetrated to the outer surface of the eye. The course of events was not followed beyond this point, the experiments being terminated on account of the tendency to hemorrhage and secondary infection of the exposed tumor mass.

In the group of animals studied, there was only one which showed signs of permanent regression of the ocular tumor during a period of 3 to 6 weeks observation. The tumor in this instance developed from the corneal incision and was located chiefly in the conjunctival tissues. On the other hand, no metastases were demonstrated in any of these animals.

Tumors in the brain grew very rapidly, exhibiting a tendency to spread rather diffusely along the surfaces of the meninges and over the walls of the ventricles, and in most instances, there was an abundant growth about the choroid plexus. Of the five animals comprising this group, one died 12 days after inoculation and another on the 16th day as a result of a growth in the cranial cavity. The three remaining animals showed pronounced symptoms of intracerebral pressure and were killed on the 17th day. None of these animals showed visible metastases, and it was apparent from this experiment that rabbits inoculated in the brain would rarely survive longer than 3 weeks.

The growth of tumors in the muscles was also rapid. Irregular lobulated masses or multiple nodules developed at the site of inoculation, and within 2 to 3 weeks, some of these reached a size of from 1 to 3 cm. in diameter. They invaded the muscle and extended centrally for a considerable distance, but in the small group of animals studied thus far, all the primary tumors have undergone spontaneous regression, and with one possible exception, no distant metastases have been found.[1]

The tumors produced by intracutaneous inoculation were usually small and rarely exceeded 1 cm. in diameter. They were smooth and elastic, and at the height of their activity, not infrequently invaded the surrounding skin and showed surface ulceration. The fate of these tumors was variable. In animals inoculated on the sheath only, the tumors persisted for a comparatively short time and no metastases were observed, but the skin tumors of a few animals

[1] The animal of this series which showed the most persistent growth was subsequently inoculated with *Treponema pallidum* and developed a severe generalized syphilis. At the height of this disease, the animal was given a second tumor inoculation in the subcutaneous tissues. There was a slight local growth, and when the animal was killed 6 weeks later (6 months after the first inoculation), an actively growing metastasis was found in one of the adrenals.

inoculated in both the testicles and the sheath continued to grow parallel with those in the testicle and metastases developed in the inguinal lymph nodes which may have been derived from the tumor in the skin.

With subcutaneous inoculations, there was a further reduction in the extent of the primary growth. As has been mentioned, small, intensely indurated or elastic nodules of 3 to 8 mm. in diameter developed at the site of inoculation in all animals, but by the 10th day, they usually began to soften and diminish in size, leaving only a small fibrous mass. Clinically, such cases may be regarded as negative in that the growth of tumor cells could not be distinguished from the granulation tissue which formed the greater part of these lesions.

In other instances, the growth assumed the form of an irregular elastic nodule or of a firm plaque which continued to increase in size for 2 to 3 weeks and was almost invariably associated with a marked enlargement of the regional lymph nodes as shown in Figs. 1 and 2. This feature of the reaction was at times helpful in distinguishing between small tumor nodules and masses of granulation tissue. In both cases, the regional lymph nodes became enlarged, but the enlargement was much greater when there was a definite growth of tumor cells. This condition is illustrated in Figs. 3 and 4 which are taken from two animals of the same series which were inoculated in the mammary line on the right side of the thorax. In Fig. 4, the nodule at the site of inoculation is barely perceptible and the enlargement of the adjacent lymph nodes is much less than in Fig. 3 which shows a small but definite tumor nodule.

This was the picture usually presented, and in cases of small nodules of uncertain character, the associated lymphoid reaction was helpful in arriving at a diagnosis of the probable extent of the tumor growth which had taken place. For the same reasons, inoculations in the mammary line were preferable to those made in the axilla or groin.

Subcutaneous tumors were either single or multiple; they were usually lobulated and rarely exceeded a centimeter in diameter. As a rule, regression began within 2 to 3 weeks after inoculation, but in exceptional instances, growth was more active and more prolonged and the tumors attained a size of 2 or more cm. in diameter. An

example of a growth of this type is given in Figs. 5 to 8 which represent stages in the development of a tumor from 9 to 37 days after inoculation. An interesting feature of the condition here shown is the reduction in the size of the inguinal lymph nodes which took place during the later stages of tumor growth or preceding any appreciable degree of regression.

Thus far, no metastases have been found from primary tumors in the subcutaneous tissues of normal rabbits; all of them have undergone spontaneous resolution, and 4 to 5 weeks was the extreme limit of their activity. This fact should be emphasized, since in certain classes of syphilitic rabbits, inoculation by this method will lead to the development of metastases and to the death of the animal.

Little can be said of the growth which takes place after intraperitoneal or intravenous inoculation. In the two instances of this kind in our series, the growth was generalized, and one animal (intravenous) would undoubtedly have died, but the probable fate of the other is uncertain.

In general, primary tumors of the eye, the muscles, the skin, and the subcutaneous tissues showed periods of increased and decreased activity analogous to those described in tumors of the testicles, and in the case of the most active growths, resolution not infrequently began abruptly and at times was preceded by an acute swelling or edematous infiltration of the surrounding tissues.

In spite of the frequency of negative results and the relatively benign character of the growth obtained by intramuscular, intracutaneous, and subcutaneous inoculations, animals inoculated by these routes developed an immunity which was sufficient to protect them from subsequent inoculation by the same route or from inoculations made into the testicles.

The outstanding features of this group of experiments were the varying incidence of primary tumors by different methods of inoculation, the relatively benign character of the growth in the muscles, the skin, and the subcutaneous tissues and the constant occurrence of spontaneous resolution, the absence of metastases, the persistence of the growth in the eye, and the rapidly fatal course of tumors in the brain.

Inoculations with Cells Repeatedly Frozen and Thawed.

It has been observed that comminution of tumor cells, as by grinding, may reduce their activity, and that while the cells survive outside of the animal body for long periods of time at low temperatures, the success of transplantation is usually diminished by repeated freezing and thawing.

In our earlier experiments, it was found that inoculations made with a cell emulsion prepared by grinding the tissue in a mortar gave results which were fully as good as those obtained by the use of small pieces of tumor tissue. This suggested an unusual degree of resistance to mechanical influences, and in order to test this condition further, a series of experiments was carried out with a cell emulsion which had been subjected to repeated freezing, thawing, and grinding.

Method.

The method used was as follows: A cell emulsion was prepared as for ordinary inoculation and divided into two parts, one of which was placed in the ice box for the inoculation of control animals. The other portion was placed in a flask and reduced to a temperature of −20° to −25°C. and kept at this temperature for 30 to 40 minutes. The emulsion was then thawed rapidly by immersion of the flask in warm water and the process repeated. Finally, the frozen material was placed in a mortar and ground before inoculation, and a dark-field examination of the emulsion was made to determine the extent to which the cells had been broken up. Inoculations were made intratesticularly, as usual, and controlled by the inoculation of a second series of animals with the portion of the original cell emulsion which had been kept in the ice box.

Two experiments of this kind were done. In the first experiment, the emulsion was frozen twice before being inoculated into five rabbits. One of these was killed 8 days after inoculation in order to determine microscopically whether there was any evidence of a growth. The four other animals developed tumors after an incubation period of between 2 and 3 weeks as contrasted with 1 week in the controls.

In the second experiment, the emulsion was frozen and thawed five times, but the results were essentially the same as in the first. The incubation period was slightly longer, but all the animals developed tumors.

These experiments show a remarkable resistance on the part of the agent responsible for the transmission of the tumor, but may be explained upon the basis of a failure to destroy the tumor cells, since dark-field examination of the emulsion used in the second experiment showed that while the majority of the cells were fragmented, there were a few that presented an apparently normal appearance.

Inoculations with Desiccated Tumor Tissue and Cell-Free Filtrates.

With a view to determining whether the tumor could be propagated by inoculation of material which contained no living tumor cells, three series of experiments were carried out with desiccated tumor tissue and with cell-free filtrates.

Methods.

Desiccation of the tumor tissue was accomplished by spreading a thin layer of tumor pulp over the bottom of a large Petri dish, freezing, and drying in a vacuum over phosphorus pentoxide while in a frozen condition. An emulsion was prepared from the powder obtained and injected as usual.

The filtrates were prepared from an ordinary cell emulsion, diluted slightly more than usual, by passing through fine, medium, or coarse Berkefeld filters.

Inoculations were made in the testicles, the anterior chamber of the eye, and the brain. The activity of the growth used for the preparations of these materials was controlled in each case by intratesticular inoculation carried out with the fresh cell emulsion.

All these experiments gave negative results. While this does not prove that living tumor cells are essential to the propagation of the tumor, the experiments do show that the causative agent was not capable of passing even a coarse Berkefeld filter or of surviving the process of desiccation as was the case with the chicken tumors studied

by Rous and his collaborators (3). The possibility of the existence of an agent distinct from the tumor cell has not been investigated beyond this point.

Inoculation of Foreign Species.

A few attempts were made to transmit the growth to rats and to guinea pigs by intratesticular and intracerebral inoculation but with negative results in all cases.

DISCUSSION AND CONCLUSIONS.

Taken as a whole, the most striking feature of the behavior of the transplanted tumor, as recorded in this and the preceding paper (2), is the wide difference in the results which may be obtained by varying the method of inoculation. The fact that, under one set of conditions, the tumor is practically incapable of growth, while, under another, it is capable of producing a most malignant disease, and, under still other conditions, produces a growth of considerable proportions but of relatively benign character, affords an exceptional opportunity for the study of phenomena of tumor growth and of conditions which contribute to malignancy or to resistance on the part of the host. It is not our intention, however, to discuss these subjects at this time further than to point out certain features of the experiments reported which indicate the sphere of action of several groups of factors which determine the results of transplantation.

It is obvious that the tumor possesses a capacity for growth which is not exhibited by the cells of normal tissues, and that, under favorable conditions, it is capable of producing a disease which is analogous in many respects to human cancer. It is apparent, however, that neither the extent of the growth nor its effects upon the animal organism are determined solely by the activity of the tumor cells, but that there are a number of factors which contribute to these results, all of which are effective either by direct action upon the tumor cells or through the intervention of the host. In brief, there are three elements which affect all experimental work of this kind. These are the material used, the method, and the animal. The first two of these factors can be maintained at a fair level of constancy

or varied almost at will provided the investigator is sufficiently familiar with his material. Still, the capacity for growth of a given material can be estimated only within certain limits, and at times results will be obtained which can be explained only upon the assumption of a capacity for growth which was much greater or much less than had been supposed. The third factor, however, is the most uncertain of the group. Its value is difficult to estimate and is subject to change, hence an intimate knowledge of the animal is the most important element in the experimental equation. In the experiments reported, there are numerous examples of the influence of each of these factors in determining the results of tumor inoculation.

The capacity for growth possessed by the cells of this tumor is brought out to greatest advantage by inoculations made into the brain, the eye, and the testicle, where large and rapidly growing tumors are produced in almost every animal. When given the advantage of such locations as the brain, the testicle, the anterior chamber of the eye, the muscles, or even the skin of the sheath, growth of the tumor cells stands out in practically all animals as an aggressive and dominant force for a period of from 2 to 4 weeks. During this time, only the exceptional animal appears to be capable of opposing an effectual resistance.

In the subcutaneous tissues, conditions are reversed. Native resistance is sufficient to dominate the situation completely. The tumor grows very little if at all in most animals, and it is only the one animal in four or five whose resistance is so feeble that the tumor is able to reach any considerable size. Even in these cases, however, growth is arrested before metastases develop, and no serious consequences are produced. Obviously, similar results might be brought about by nutritional deficiencies as in the case of ill nourished, old, or diseased animals, but in the present instance, the contingency of a passive nutritional deficiency can be elimiñated on account of the ease with which metastases grow in both the skin and subcutaneous tissues of animals whose resistance has been broken. Given this advantage of location, therefore, animals with the least resistance are able to confine the activity of the tumor cells to essentially the same plane as that reached in the most resistant animals inoculated

in the testicles or far below that of intracerebral inoculations. In other words, by merely varying the location of the tumor graft, extremes of resistance may be equalized or made to overlap. Apparently these differences in results are due to the opportunity afforded for bringing the forces of resistance into operation or are matters of advantage and disadvantage.

With the growth of tumors in any location, the resistance of all animals tends to increase, and apparently the extent of the increase varies according to the demand, within the capacity of the individual animal, and, except in the case of tumors in the brain, a point is usually reached at which resistance becomes dominant. In the brain, the extent of the local growth almost inevitably causes death before there is any opportunity to check its progress (2 to 3 weeks). With other forms of inoculation, there is either a temporary arrest or complete suppression of growth, and here again the result is determined in part by the capacity of the individual animal and in part by the advantage given through inoculation. In the case of tumors of the eye, the end-result is unknown, but with tumors in the skin and muscle, complete suppression occurs, and with possible rare exceptions, the growth is controlled before metastases develop.

With testicular tumors, conditions are different. In a single series of ten or twelve animals, one may see every degree of antagonistic activity between the forces of growth and of animal resistance included between the extremes of a slight and transient primary growth and a fulminating case of malignancy. A few animals possess a high threshold resistance which is sufficient to reduce the growth of the tumor to an almost negligible quantity. In a somewhat larger group of animals, the initial resistance is comparatively low, and the tumor grows rapidly, but there is a prompt and efficient reaction on the part of the animal with suppression of growth as by crisis. The opposite of this condition is also seen in animals with slowly growing but rather persistent tumors. Apparently these animals have a relatively high native resistance but are either incapable of a more vigorous reaction or are not aroused to their full capacity. All of these are examples of either a high threshold resistance or a reserve capacity for prompt and vigorous reaction.

At the other end of the scale, there are a few animals with neither native resistance nor reserve capacity sufficient to offer more than a

feeble opposition to the growth of the tumor cells. These animals furnish the fulminating cases of malignancy and occur in a proportion of about one in five in any group of rabbits, or in the same proportion as animals in which the tumor grows actively from inoculations made into the subcutaneous tissues.

Between the two extremes, the opposing forces are more nearly equal, and the balance swings first in one direction and then in the other. A study of the conditions presented by this group of animals is especially instructive. It is obvious that of the two sets of factors concerned in these reactions in a given experiment, the potentialities for growth represented by the tumor are relatively fixed and the source of variation is to be sought in the host.

The renewal of growth in a tumor after a period of inactivity or regression raises the question as to whether this is due to a passive decline in the resistance of the animal which follows a temporary arrest of the growth or is a phenomenon of exhaustion. As has been pointed out elsewhere, periodic changes of this kind are not peculiar to either spontaneous or transplanted tumors but are phenomena characteristic of many diseases, especially those which pursue a chronic course, and in all these conditions the outcome would appear to be determined by the way in which the host responds as well as by the ability to meet the demands which are imposed upon it.

There are many circumstances connected with the growth of this tumor which suggest that the effort put forward by the animal is roughly proportional to the demand. One such instance has been cited in connection with the suppression of rapidly growing and of slowly growing tumors. But apparently this does not hold in all cases. The fact that the animals composing the group in which there is a shifting balance gradually become separated into two subgroups one of which eventually controls the tumor while those of the other succumb, suggests that in one instance the occurrence of periodic changes may be due to a lack of sustained effort or a passive decline in resistance following a temporary suppression of growth, while in the other, there is clearly a failure in the ability of the animal to maintain an effectual opposition to growth which is suggestive of exhaustion.

Going a step further, there is evidence to show that a close relation exists between the local expression of resistance to tumor growth

and the general health of the animal. For example, animals that control their tumors readily show an improvement in physical condition and are not only immune to further inoculation with the tumor but are highly resistant to epizootic infections and to syphilitic infections as well, while constitutional symptoms of disease and feeble resistance to the same class of influences are associated with a defective or feeble resistance to tumor growth.

Moreover, the development of cachexia and the occurrence of death are not necessarily related to either the mass of the tumor or the rate of its growth but to the ability of the animal to confine the growth to a given location. Large primary tumors may produce practically no constitutional symptoms, and animals with large metastatic tumors may remain in excellent condition for months, but with steadily progressive tumors or when showers of metastases appear in the skin, subcutaneous tissues, and other parts of the body at an early period, there is a gradual or rapid decline in physical strength, as the case may be, followed by death, even though the primary tumor may be comparatively small and the total mass of tumor tissue no greater than is frequently found in animals that show no symptoms of disease. In the one instance, the picture presented is that of a slow exhaustion, while in the other, it approaches the condition of a sudden collapse.

In the same way, one finds in any group of apparently healthy and vigorous animals a relatively uniform threshold resistance, while the reserve capacity of these animals varies widely as regards their resistance to tumor growth or to other noxious agencies, and it is the reserve capacity which forms the true measure of their resistance.

In other words, there is a close parallelism between the functioning of those processes which are concerned with the limitation of tumor growth and those which have to do with the maintenance of the general health of the animal. At any rate, each of these processes is conditional upon the other, and both are dependent to some extent upon the same mechanism. In its final analysis, therefore, it is apparent that while the quality of malignancy as brought out in these experiments is conditioned upon the capacity for growth of the tumor cells, the degree of malignancy displayed in any given instance is a function of animal resistance which is not entirely

specific but corresponds closely with the ability of the animal to meet excess demands arising from other causes and, to this extent, is an expression of the functional capacity of a mechanism which is concerned primarily with the maintenance of the general animal economy.

These distinctions as to the sphere of influence of the several groups of factors in determining the results of tumor growth, the manner in which individual animals respond to the presence of the tumor, and the relation of these reactions to the effects of the tumor upon the animal organism are important in that they furnish a basis for an experimental approach to problems of tumor growth and malignancy, and the conceptions which have been outlined above will be developed more fully in future papers dealing with metastases, conditions which influence malignancy, and effects produced by the tumor upon the animal organism.

SUMMARY.

The results of seven methods of transplantation of a malignant tumor of the rabbit are reported and contrasted with results obtained by intratesticular inoculation.

It was found that inoculations made into the brain, the anterior chamber of the eye, and the muscles gave an almost uniform series of takes with the production of comparatively large and vigorously growing tumors. Intracutaneous inoculations were less favorable, while with subcutaneous, intravenous, and intraperitoneal inoculations, a growth was obtained in only 20 to 25 per cent of the animals.

In contrast with the malignant course of the disease produced by testicular transplantation, the tumors produced by methods other than intracerebral inoculation were relatively benign. In all cases, the growth was of a purely local character; in some instances, there was invasion of the surrounding tissues with local or regional extension of the growth, but no metastases developed in distant organs. Tumors in the brain soon caused death as a result of pressure, and the ultimate fate of tumors in the eye was not determined, but the growth produced by other methods of local inoculation was of relatively short duration and terminated with spontaneous resolution.

It was found that while a good growth could be obtained with material which had been subjected to repeated freezing, thawing, and grinding, the use of cell-free filtrates or desiccated tumor tissue gave negative results.

In discussing the experiments reported, it was pointed out that there were three groups of factors which had played important parts in determining the results of transplantation. These were the capacity for growth of the tumor cells, animal resistance, and the advantage given the tumor or the animal by the use of a particular method of inoculation. From an analysis of the results obtained with reference to the operation of these factors, the conclusion was reached that while the quality of malignancy displayed by the tumor was conditioned upon the capacity for growth of the tumor cells, the degree of malignancy exhibited in any given instance was a function of animal resistance which was not entirely specific but corresponded closely with the ability of the animal to meet excess demands arising from other causes and, to this extent, represented an expression of the functional capacity of a mechanism which is concerned primarily with the maintenance of the general animal economy.

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EXPLANATION OF PLATES.

All the illustrations are reproductions of photographs which have not been retouched. Objects are represented at their natural size. Statements of time are estimated from the day of inoculation.

PLATE 44.

FIG. 1. 9 days. Actively growing primary tumor in the subcutaneous tissues with marked enlargement of the inguinal lymph nodes.

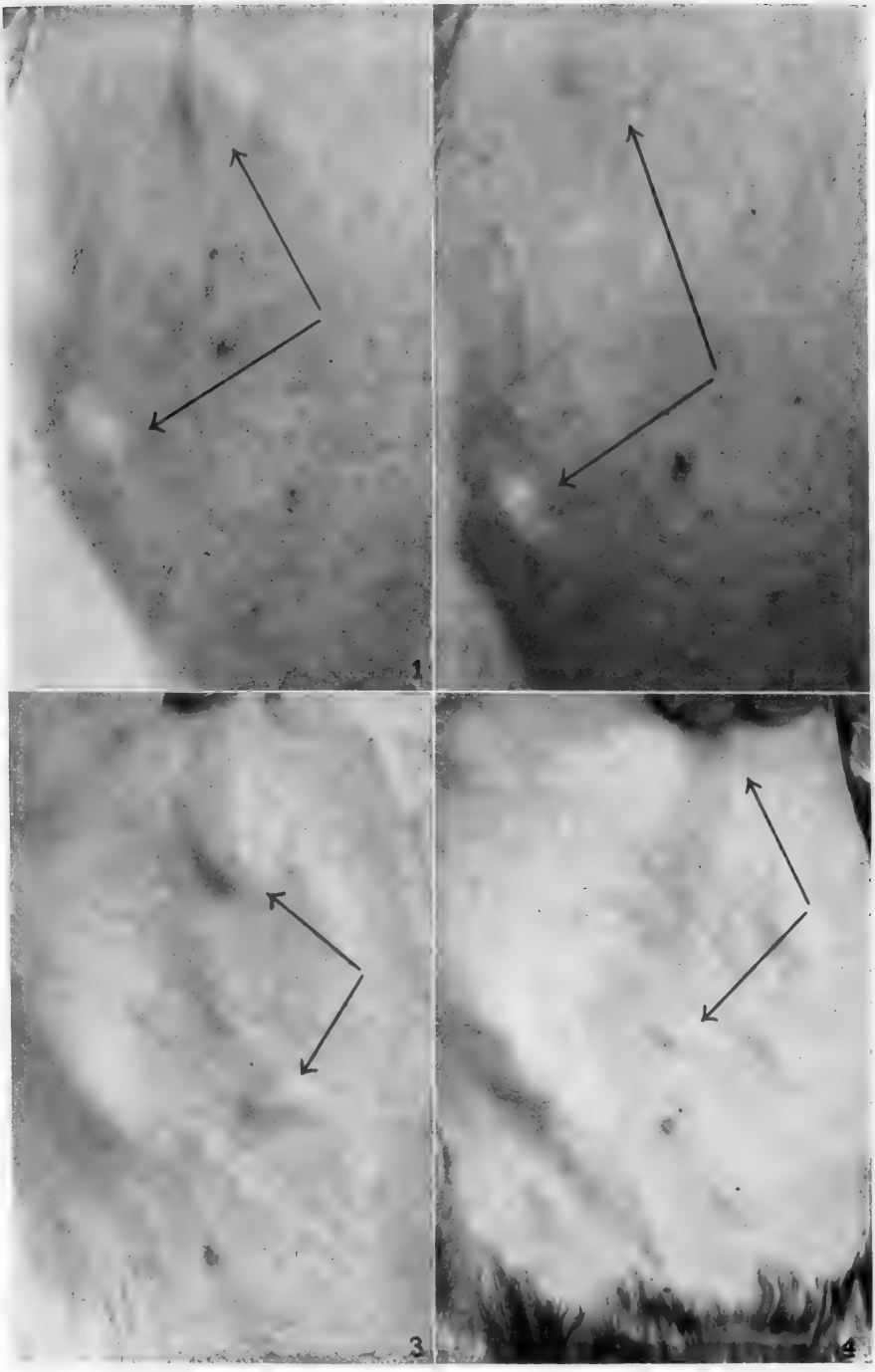
FIG. 2. 9 days. A flattened type of primary growth in the subcutaneous tissues, comparable in all other respects with that in Fig. 1.

FIG. 3. 9 days. A small nodular growth in the subcutaneous tissues of the upper mammary region with enlargement of the mammary and axillary lymph nodes.

FIG. 4. 9 days. A negative result in an animal of the same series as that in Fig. 3. Note the small nodule of fibrous tissue at the site of inoculation (marked by an arrow) and the absence of any marked enlargement of the drainage lymph nodes.

PLATE 45.

FIGS. 5 to 8. 9, 14, 23, and 37 days respectively. Stages in the development of an unusually large and persistent tumor in the subcutaneous tissues. Note the relatively slow growth during the first 2 weeks as compared with the growth during the 3rd week and the reduction in the size of the inguinal lymph nodes between the 3rd and 5th weeks. The slow initial growth of the tumor may also be seen by comparing Figs. 1 and 5.



(Pearce and Brown: Malignant tumor of the rabbit. IV.)

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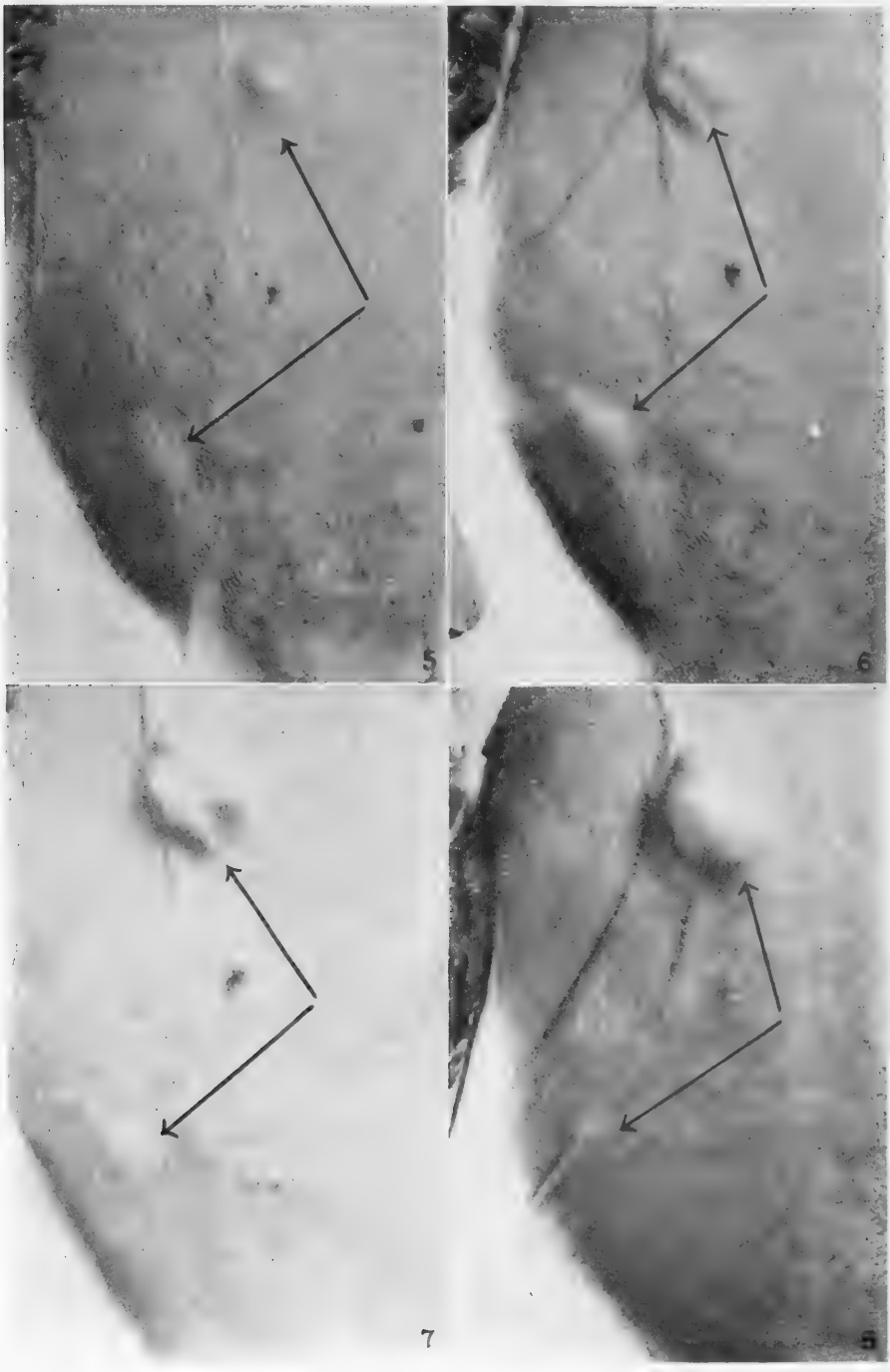

(Pearce and Brown: Malignant tumor of the rabbit. IV.)

THE DEPOSITION AND SUBSEQUENT COURSE OF PARTICULATE MATERIAL (MANGANESE DIOXIDE AND MANGANESE META-SILICATE) ADMINISTERED INTRAVENOUSLY TO CATS AND TO RABBITS.

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INTRODUCTION.

In 1920, Reiman and Minot (1) showed that manganese could be quantitated accurately and readily in animal tissues even when in high dilution. It then occurred to us that if manganese compounds of relatively insoluble type could be found—substances which permitted a high degree of subdivision and suspension without agglutination in liquids suitable for animal injection—useful quantitative experiments upon the deposition and excretion of foreign particles could be accomplished. Our principal interest in such research was concerned with the idea that a more exact study of lung fibrosis due to foreign material might be made if dusts susceptible to chemical quantitation were employed, and we contemplated, at first, a series of investigations upon animals exposed to atmospheres containing different manganese dusts. There are, however, great objections to the inhalation method of getting foreign material deep into the lungs. It is not only difficult to get the dust in, but it is impossible to estimate the amount of dust which, at any given exposure, actually reaches the region of the alveoli. Even, therefore, if one does employ a dust which can be quantitated with the greatest accuracy in animal tissues, one cannot have any assurance, during the period of dusting, of the actual state of affairs in the depths of the animal's lungs.

It has, however, been shown (2) that particulate material given intravenously to cats is immediately arrested in certain characteristic positions and in amounts which, within rough limits, can be relied upon. Thus, 1 hour following intravenous injection of a suspension of manganese dioxide, 47 per cent of the injected material could be recovered from the lungs, 38.3 per cent from the liver, 4.3 per cent from the spleen, and the remainder was scattered through the gastro-intestinal tract, bones, kidneys, etc. It is, therefore, possible to deposit particles averaging 1μ in diameter in the lung capillaries. This site of initial deposition is upon the blood side of the lung parenchyma, in contrast to what may be called the normal site of particle deposition; namely, upon the alveolar or air side of the lung tissue. Histological study of manganese dioxide arrested intravascularly showed that part of this material passed out of the vessels into the alveolar walls, and, having made this transit, occupied the position which would be taken by inhaled particles carried into the lung tissue from the alveoli. This being the case, it became our problem to test different compounds of manganese deposited in the lung by intravenous injection, in terms of their future passage through the animal. If we were to be justified in an attempt to produce lung fibrosis in cats by means of repeated small intravenous injections of different compounds, it became necessary, first of all, to determine the fate of large single injections.

This paper, therefore, deals with findings in the following groups of experiments.

I. (*A*) Quantitation of manganese in the organs of cats which had received intravenous injections of manganese dioxide 3 to 168 hours previously. (*B*) A similar series of experiments upon rabbits.

II. Quantitation of manganese in the organs of cats and of rabbits which had received intravenous injections of rhodonite (manganese meta-silicate). These experiments include (*A*) injections of rhodonite of three different sizes into cats, followed by analyses of organs 1 hour later, and (*B*) injections into cats of suspensions of rhodonite particles averaging 1.1μ in diameter, with analyses of organs 1 to 55 days later, and into rabbits, with analyses of organs 1 to 16 days later.

EXPERIMENTAL.

I. The Fate of Manganese Dioxide Administered Intravenously.

A. Cats.—In all the experiments with manganese dioxide the suspensions used for injection, the technique of the injections, and the method of quantitative analysis for manganese were the same as those used by Drinker and Shaw (2) and are described in detail in their paper. It is sufficient, here, merely to recall the fact that these injections consisted of a suspension of powdered, chemically pure manganese dioxide containing no particles or aggregates of particles larger than 1μ suspended in an acacia-sodium chloride solution.

TABLE I.

Percentage of Manganese Recovered from the Lungs and Liver of Cats Injected 1 to 168 Hours Previously with Manganese Dioxide.

Tissue.	Length of time after injection.									
	1 hr.*	3 hrs.	6 hrs.	12 hrs.	18 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.	168 hrs.
	Average of 20 experiments.	Average of 2 experiments.	Average of 3 experiments.	Average of 3 experiments.	Average of 2 experiments.	Average of 2 experiments.	Average of 2 experiments.	Average of 2 experiments.	1 experiment.	1 experiment.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Lungs.....	47.0	42.7	29.4	20.0	2.9	7.2	1.4	0.2	0.1	0.1
Liver.....	38.3	47.5	52.8	55.0	56.8	44.3	19.3	14.8	5.3	3.6
Total.....	85.3	90.2	82.2	75.0	59.7	51.5	20.7	15.0	5.4	3.7

* The figures for the 1 hour experiments are taken from Drinker and Shaw (2), p. 87.

Table I gives the average percentages of the original injections of manganese recovered from the liver and lungs of a series of cats injected intravenously 1 to 168 hours previously with manganese dioxide. In cases of survival longer than 3 hours, the injection was made under ether or under local anesthesia. On the hour terminating the experiment, the animals were anesthetized with ether and bled to death. In the short period experiments, urethane anesthesia was used and the animals were bled to death while under this anesthetic. The size of the injections ranged from 3.92 to 10.68 mg. of manganese, the average injections being 6.4 mg. In each instance the normal

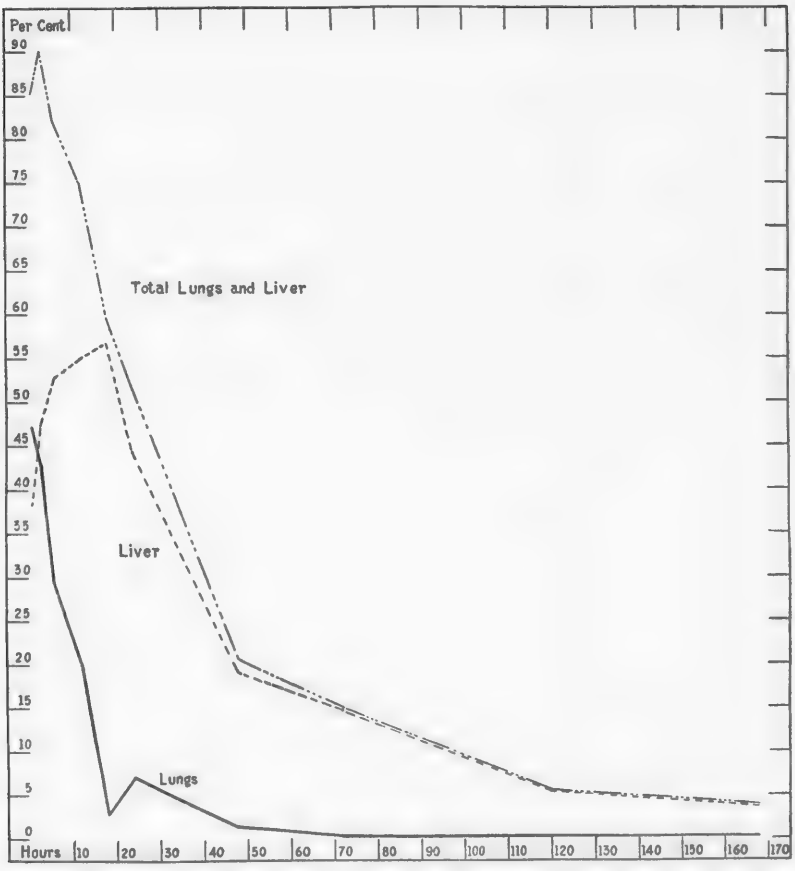
manganese content of the organ was subtracted from the amount of manganese recovered before the percentage recovery was estimated. Unless indication is made to the contrary, this is true of all percentage recoveries reported in this paper. The figures in Table I illustrate the initial large deposition of manganese in the lungs of the cat, the prompt transference of the lung manganese to the liver, and the gradual elimination of manganese from the body. Analyses of the other organs and tissues of the body, 168 hours after injection of manganese dioxide, gave practically negative results. The liver was the only organ which contained more than 0.3 per cent of the original injection.

Text-fig. 1 illustrates graphically the figures and facts given in Table I. The chief points of interest in this chart are the rapid rise in the height of the liver curve, corresponding to the fall in the curve of percentage manganese in the lung, and the fact that after the first 20 hours the liver curve and the curve of total recovery from the lungs and the liver practically coincide.

B. Rabbits.—Lund, Shaw, and Drinker (3) have shown that manganese dioxide, given intravenously to the rabbit, rat, guinea pig, dog, chicken, and turtle, is immediately arrested in the liver, practically to the exclusion of other organs. To this group the monkey can now be added. Owing to the fact that in the cat there is an apparent transfer of manganese dioxide from lungs to liver, it was decided to follow the rate of elimination in the rabbit, in which the entire process is carried on without lung intervention.

Table II summarizes a series of experiments on rabbits similar to those on cats shown in Table I. The figures in this table are the percentages of the original injections of manganese recovered from the lungs and liver of rabbits injected 1 to 120 hours previously with manganese dioxide. The material injected varied in amount from 2.71 to 10.475 mg., the average being 5.548 mg. of manganese. In this table figures from individual experiments are given, not averages from several experiments, as in Table I. The purpose of this is to illustrate the normal variation in rate of excretion which occurs among different animals of the same species.

The principal facts which Table II illustrates are the small initial deposit of manganese in the lungs of rabbits, the very large initial



TEXT-FIG. 1. Percentage of manganese recovered from the lungs and liver of cats injected 1 to 168 hours previously with manganese dioxide.

TABLE II.

Percentage of Manganese Recovered from the Lungs and Liver of Rabbits Injected 1 to 120 Hours Previously with Manganese Dioxide.

Tissue.	Length of time after injection.																					
	1 hr.*	1 hr.*	1 hr.*	3 hrs.	3 hrs.	6 hrs.	6 hrs.	12 hrs.	12 hrs.	18 hrs.	18 hrs.	24 hrs.	24 hrs.	36 hrs.	48 hrs.	48 hrs.	72 hrs.	72 hrs.	96 hrs.	96 hrs.	120 hrs.	120 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Lungs	1.2	0.2	0.5	1.0	0.8	3.1	0.7	0.9	1.5	0.3	0.3	0.8	0.4	0	0.3	0.2	0.4	0.2	0	0.1	0	0
Liver	81.0	81.0	85.6	68.2	84.6	42.4	17.6	20.3	11.4	17.0	16.4	28.7	20.0	2.4	2.2	0.3	3.7	3.6	5.1	1.6	0.8	0
Total	82.2	81.2	86.1	69.2	85.4	45.5	18.3	21.2	12.9	17.3	16.7	29.5	20.4	2.4	2.5	0.5	4.1	3.8	5.1	1.7	0.8	0
Average totals	83.2			77.3		31.9		17.1		17.0		25.0		2.4	1.5		4.0		3.4		0.4	

* The figures for the 1 hour experiments are taken from Lund, Shaw, and Drinker (3), p. 234.

deposit in the liver, the individual variations which occur among animals of the same species in rate of excretion, and the steady elimination from the body of the rabbit of intravenously injected manganese dioxide, an elimination more rapid than in the cat because of the fact that the rabbit lung plays practically no part in the initial removal from the blood stream of intravenously administered manganese dioxide.

II. The Fate of Manganese Meta-Silicate (Rhodonite) Administered Intravenously.

Manganese Meta-Silicate Suspensions.—The suspensions used for injection in the manganese meta-silicate work were made from crushed rhodonite obtained from Franklin, New Jersey.[1] Rhodonite, or fowlerite as the light-colored form of rhodonite obtained from Franklin is called, consists of a mixture of large, rough, red and pink crystals and of granular or rounded separate grains of manganese meta-silicate ($MnSiO_3$). Selected crystals of rhodonite, obtained from the sorting tables at Franklin, were first crushed to a coarse powder and then forwarded to us. This coarse material was then reduced to a fine powder in a ball mill. It was next washed in distilled water until the supernatant liquid after centrifugalization showed no visible foreign particles (approximately five to six washings).

Particles of the desired size were sorted out by the process of settling. This was accomplished by adding distilled water to rhodonite powder and decanting the mixture into a large wide mouthed bottle. The bottle was thoroughly shaken and the rhodonite allowed to settle until particles larger than those desired had fallen below a given level. Suspension A^1, for example, was obtained by allowing the undifferentiated suspension to settle for 3 hours. A glass tube with one end turned to form a U and the other end attached to a suction bottle was then introduced at a point 1 inch below the surface and the overlying suspension was drawn off. The stock bottle was again shaken and the process of settling and draining off of the upper layer of liquid repeated until a sufficient quantity of suspension containing particles of the desired size was obtained.

The length of time during which each suspension was permitted to settle was determined originally by microscopic examination of the layer of liquid to be withdrawn, and was increased or shortened depending on whether larger or smaller sized particles were desired. In any event, the supernatant liquid always contained not only the largest sized particles desired but also smaller particles, ranging down to those of ultramicroscopic size. The size of particles referred to in our suspensions, therefore, indicates the size of the largest particles present.

[1] The crushed rhodonite was obtained through the courtesy of the New Jersey Zinc Company.

Suspension C was obtained, not by the process of settling, but by centrifugalization of a suspension of rhodonite in distilled water. As an extremely fine suspension was desired, centrifugalization, rather than settling, was employed in order to save time. Particles of rhodonite suspended in distilled water will remain discrete for an indefinite period provided they are kept at a temperature of about 2°C.

The terminology employed in referring to the suspensions in the experiments may be explained as follows:

Suspension A^1.—This contained all particles which settled at a rate of not more than 1 inch in 3 hours. The maximum size of these particles, calculated theoretically from the density of rhodonite (3.62) and from the speed of settling, was 1.3μ (4).

Suspension B^1.—This suspension, obtained from the same stock suspension as A^1, contained all particles which settled at a rate of not more than 1 inch in 20 hours. Calculated theoretically, the maximum size of these particles was 0.5μ.

Suspension C^1.—The supernatant liquid obtained by centrifugalizing for 3 minutes at high speed the original stock suspension of rhodonite from which Suspensions A^1 and B^1 were taken, was designated C^1. The largest sized particles in this suspension were just visible microscopically and exhibited active brownian movement. It is safe to conclude that Suspension C^1 contained no particles above 0.2μ in diameter.

Suspension A^2.—The discovery of the presence of traces of soluble manganese in Suspensions A^1, B^1, and C^1 after they had been used in a brief series of experiments led to the preparation of a fourth suspension, A^2, which, before the process of settling was undertaken, was washed with nitric acid in order to remove the readily soluble manganese, and finally with distilled water until the wash water was neutral to litmus. Suspension A^2 contained all particles which settled at a rate of not more than 1 inch in 4 hours. The maximum size of the particles in Suspension A^2, calculated theoretically, was 1.1μ. Four 5 cc. samples of Suspension A^2, dried to constant weights and analyzed chemically, showed the following percentages of manganese: 17.42, 16.94, 17.76, 17.58—an average of 17.43 per cent.

With the exception of a brief series of experiments with Suspensions A^1, B^1, and C^1, in which the deposition in the body of different sized particles was compared, Suspension A^2 was used in all the rhodonite work.

The amount of rhodonite injected varied from 0.9 to 2.1 mg. per kilo of body weight, the concentration from 0.137 to 0.328 mg. of manganese per cc. of injection. With the exception of Suspension C^1, the volume of the various rhodonite injections ranged from 20 to 25 cc. of suspension. Owing to the difficulty of concentrating Suspension C with its very fine particles, either by settling or by centrifugalization, the concentration of this suspension averaged only 0.0714 mg. of manganese per cc. of suspension. For this reason, 60 cc. of this suspension were injected. In all instances, the suspensions were made physiologically saline with sodium chloride before introduction into the animal.

The actual injection of the suspension, as with manganese dioxide, required 2 to 3 minutes and never caused the least toxic effect. Injected animals remained in good health for indefinite periods.

Organs obtained immediately after the animal had been bled to death were analyzed quantitatively by a modification of the Reiman-Minot method of manganese determination. Since a description of this modified method has never been published, it seems worth while to include at this point a brief account of it.

Technique of Manganese Meta-Silicate Determination.—Tissues to be analyzed are carefully cleaned of adhering bits of fat and connective tissue and are weighed as promptly as possible after removal from the animal. In the present experiments wet weights were used throughout. After being weighed, tissues are placed in small porcelain evaporating dishes and are dried from 12 to 36 hours in a gas oven, the time depending on the size of the tissue. The dishes are then transferred to an electric furnace which is gradually raised to a temperature of 700–1000°C. and maintained at this point until all carbon in the dishes has disappeared (usually 1 to 2 hours). Occasionally a certain amount of graphitic carbon remains even after prolonged heating in the furnace. If the amount of graphite is more than trifling, it is readily oxidized by the addition of a few crystals of a mixture of sodium and potassium nitrates, with subsequent heating over a Tirrill flame.

From this point until the manganese salts have been converted to sulfates, the procedure departs from the original Reiman-Minot method of manganese determination—a departure necessitated by the fact that the substance is a silicate instead of an oxide of manganese.

The ashed tissue is now fused with an equimolecular mixture of sodium and potassium carbonates. On completion of the fusion, 20 to 30 cc. of hot distilled water are added to each dish in order to dissolve the excess sodium and potassium carbonates and the soluble alkaline silicates present, as well as the water-soluble chlorides, the phosphates, and the sulfates originally present in the tissues. When cooling has occurred, 50 per cent hydrochloric acid is added very slowly from a pipette to each dish until effervescence ceases. The dishes are then heated on a sand bath until no further evolution of carbon dioxide occurs on the addition of acid.

The material in the porcelain dishes is next transferred, with the aid of hot distilled water, to quartz beakers (preferably of 150 cc. capacity). To each beaker are added 5 cc. of concentrated sulfuric acid and the mixture is heated in a hood until there are left in the beaker only a few cubic centimeters of a clear fused material at a cherry-red heat. This evaporation may be effected over a Tirrill burner or, preferably, with a Bunsen burner, ring form, in order to prevent bumping. The completion of the acid fusion eliminates chlorides from the material to be analyzed and leaves the manganese in sulfate form.

With one or two minor exceptions, from this point on the procedure is the same as that described by Reiman and Minot (1). Since the concentrations of manganese present in the tissues in the present experiments have greatly exceeded those with which Reiman and Minot were working, we have had to use more ammonium persulfate in bringing about the final color reaction—3 to 4 gm. as against their 0.5 gm.

The only point of difference in our technique worth mentioning is the fact that we never used Nessler tube standards which contained more than 0.012 mg. of manganese. When the color in a sample was higher than this figure and yet not high enough to read on the colorimeter, we diluted a given portion of the sample until the color was within the range of our standards, making allowance for the dilution in our final calculations.

TABLE III.

Recovery of Manganese Following the Addition of Dried Manganese Meta-Silicate Suspensions to Horse Meat.

Sample No.	Weight of wet horse meat.	Amount of manganese added.	Amount of manganese recovered.	Percentage recovery.
	gm.	*mg.*	*mg.*	
1	20.60	1.800	1.837	102.1
2	20.51	1.794	1.831	102.1
3	20.55	1.824	1.802	98.8
4	20.50	1.791	1.860	103.9
5	20.59	1.800	1.802	100.1
Average.....				101.4

It is obvious that all reagents must be proved absolutely free from even traces of manganese before they can be used in this work.

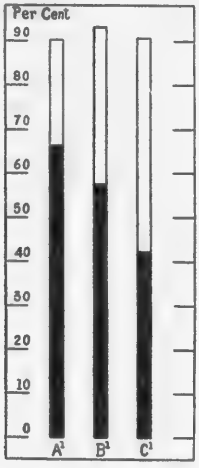
The accuracy of this method of manganese quantitation for biological work was tested by check analyses on known weights of dried rhodonite suspensions and by analyses of horse meat to which known weights of dried rhodonite suspensions had been added. The following figures are interesting in this connection. Four 5 cc. samples of Suspension A[2], dried to constant weights, contained, respectively, 0.02757, 0.02763, 0.02757, and 0.02743 mg. of solid material—a difference between the minimum and the maximum weights of 0.72 per cent. When analyzed chemically, these specimens contained, respectively, 17.42, 16.94, 17.76, and 17.58 per cent of manganese, showing a maximum departure from the mean of 2.81 per cent. As a deviation of 2.5 per cent from the mean value in making a series of colorimetric readings of a solution is permissible at this concentration, these figures show that it is possible to inject into animals accurate amounts of rhodonite, even though the injections are in the form of a suspension of insoluble particles.

In order to determine whether or not it is possible to recover, by the method of analysis used, approximately all the manganese when rhodonite is added to biological material, known weights of dried rhodonite suspension were added to 20.5 gm. samples of finely ground horse meat and the tissue was analyzed for manganese. Table III gives the results of five analyses and shows that it is possible to recover from biological material, within the limits of the accuracy of a quantitative chemical method involving a colorimeter reading as an end-point, all the manganese added to the biological material when the manganese added is in the form of a suspension of rhodonite particles.

A. Injections of Rhodonite Suspensions of Different Sizes into Cats.

A brief series of experiments consisted in the injection into cats of rhodonite suspensions of three different sizes—Suspensions A^1, B^1, and C^1—with analyses of tissues 1 hour later. These suspensions, as explained above, had not been washed with nitric acid and contained, therefore, a certain proportion of readily soluble manganese. The relative sizes of the largest particles in these suspensions were: A^1, 1.3μ; B^1, 0.5 μ; and C^1, 0.2μ. (In Suspension C^1 the majority of particles were ultramicroscopic.) The amounts of manganese injected varied from 3.3 to 4.84 mg., the average amount being 3.999 mg. The results of this work are summarized in Table IV, which gives the percentage recoveries of manganese in the lungs and liver of the cats in this series 1 hour after intravenous injection. The points to be noted in this table are the following. (*a*) The greater tendency on the part of the lungs to intercept and to retain the large sized particles. This may possibly be a function of the greater rate of speed with which large sized particles, as contrasted with smaller ones, will settle out of a moving stream, or it may indicate that the lung is less efficient than the liver in removing particles from the blood stream unless they are of considerable size. (*b*) The greater efficiency of the liver than of the lung in intercepting the smaller sized particles. (*c*) The fact that at the end of 1 hour practically the same total (90.1 to 93.3 per cent) of manganese is recovered from the liver and lungs of cats regardless of the size of the particles injected.

Text-fig. 2 illustrates graphically the facts brought out in Table IV.



■ Lung manganese.
□ Liver manganese.

TEXT-FIG. 2. Relative distribution of manganese in the lungs and liver of cats injected with manganese meta-silicate particles of different sizes. The relative sizes of the particles in the three suspensions were: A^1, 1.3μ; B^1, 0.5 μ; and C^1, 0.2μ.

TABLE IV.

Percentage of Manganese Recovered from the Lungs and Liver of Cats at the End of 1 Hour Following the Intravenous Injection of Different Sized Particles of Manganese Meta-Silicate.

Tissue.	Suspension.									Average.		
	A^1 (1.3μ). Experiment No.				B^1 (0.5μ). Experiment No.		C^1 (0.2μ). Experiment No.					
	1	2	3	4	1	2	1	2	3	A^1	B^1	C^1
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Lungs	65.0	74.1	67.7	58.3	59.5	55.9	45.4	36.4	44.5	66.3	57.7	42.1
Liver	26.1	14.4	21.6	33.1	35.0	36.1	48.5	52.3	45.3	23.8	35.6	48.7
Total	91.1	88.5	89.3	91.4	94.5	92.0	93.9	88.7	89.8	90.1	93.3	90.8

B. Injections of Suspensions of Rhodonite Particles Averaging 1.1μ in Diameter into Cats, with Analyses of Organs 1 to 55 Days Later, and into Rabbits, with Analyses of Organs 1 to 16 Days Later.

Cats.—The amounts of manganese injected varied from 4.155 to 5.04 mg. and averaged 4.435 mg. per cat. Table V gives the percentage recoveries of manganese from the lungs and liver of this series of cats, and Text-fig. 3 pictures graphically the rate at which rhodonite is eliminated from these organs. The most significant features of this chart are (*a*) the length of time which rhodonite remains in the bodies of animals injected with this substance, and (*b*) the fact

TABLE V.

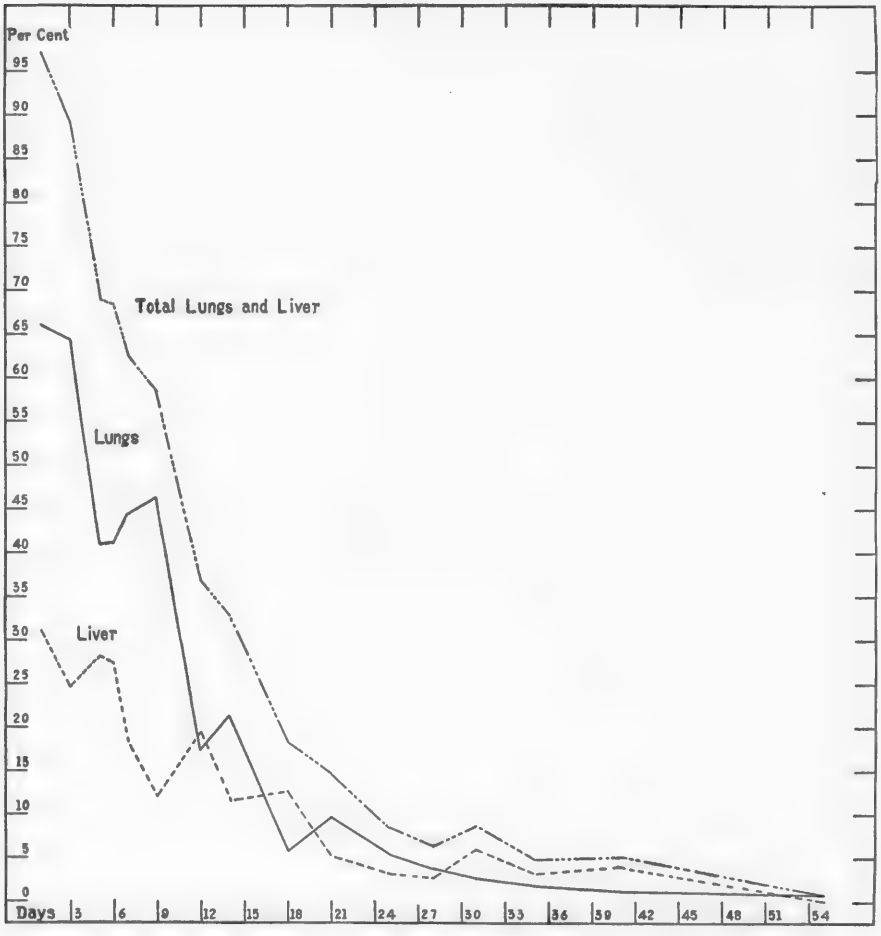
Percentage of Manganese Recovered from the Lungs and Liver of Cats during a Period of 2 Months Following the Intravenous Injection of Manganese Meta-Silicate.

Tissue.	Length of time after injection.															
	1 day.	3 days.	5 days.	6 days.	7 days.	9 days.	12 days.	14 days.	18 days.	21 days.	25 days.	28 days.	31 days.	35 days.	41 days.	55 days.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Lungs.	66.0	64.4	41.0	41.1	44.3	46.3	17.5	21.3	5.8	9.6	5.4	3.7	2.6	1.8	1.1	0.78
Liver.	31.1	24.6	28.1	27.4	18.3	12.2	19.5	11.6	12.6	5.1	3.1	2.7	6.0	3.1	4.0	0
Total.	97.1	89.0	69.1	68.5	62.6	58.5	37.0	32.9	18.4	14.7	8.5	6.4	8.6	4.9	5.1	0.78

that the curve of excretion from the liver does not rise sharply as the lung curve falls—a striking feature of Text-fig. 1, which pictures the excretion of manganese dioxide.

(*a*) At the end of 14 days a cat injected with 4.238 mg. of manganese in the form of a rhodonite suspension still retained in the lungs and liver 32.9 per cent of the original injection—21.3 per cent in the lungs and 11.6 per cent in the liver. At the end of 18 days, another cat, injected with a similar dose, still retained in the lungs and liver 18.4 per cent.

(*b*) The steep slope of the curve of manganese excretion from the lungs between the 3rd and 18th days is not compensated for by a corresponding rise in the liver curve. This suggests that although the



TEXT-FIG. 3. Percentage of manganese recovered from the lungs and liver of cats injected 1 to 55 days previously with rhodonite (manganese meta-silicate).

liver may be receiving and disposing of a certain increment of manganese from the lung, it is probably not taking care of all the material eliminated from that organ. In attempting to throw light on this point, the obvious possibility occurred to us that some of the lung manganese might be eliminated by way of the trachea. In order to test this point, analyses were made of the normal manganese content of tracheæ[2] from ten normal cats. These analyses ranged from 0 to 0.011 mg. of manganese, the average content being 0.0086 mg. Tracheæ of cats injected with rhodonite 1, 3, 5, 6, 7, 9, 18, 21, 25, and 28 days before death showed, respectively, the following manganese contents: 0.016, 0.010, 0.008, 0.006, 0.014, 0.011, 0.012, 0.030, 0.022,

TABLE VI.

Percentage of Manganese Recovered from the Lungs and Liver of Rabbits during a Period of 16 Days Following the Intravenous Injection of Manganese Meta-Silicate.

Tissue.	Length of time after injection.					
	1 hr.	5 hrs.	6 hrs.	1 day.	6 days.	16 days.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Lungs.....	1.6	2.8	1.3	6.1	0.9	0.6
Liver.....	88.2	85.2	78.7	67.4	41.7	32.4
Total.....	89.8	88.0	80.0	73.5	42.6	33.0

and 0.010 mg. of manganese, the average of these figures being 0.0139 mg. This represents an average increase over the normal manganese content of the trachea of 61.6 per cent, and suggests that some of the injected rhodonite is eliminated by way of this organ.

Rabbits.—Injections of rhodonite were made into six rabbits and the organs of these animals were analyzed for manganese after 1, 5, 6, and 24 hours, and 6 and 16 days. The amounts of material injected varied from 3.99 to 5.065 mg., the average injection being 4.476 mg. of manganese. Table VI gives the percentage findings of manganese in the lungs and liver of these rabbits.

[2] The larynx, cleared of muscle, was left attached to the trachea in all instances and its manganese content is, therefore, included in all figures given for the trachea.

DISCUSSION.

The observations reported are of interest in several directions. First, it is shown that manganese dioxide has little permanence as a foreign substance in the animal body. This finding is contrary to our original assumption in regard to this compound. Manganese dioxide is a substance which can be dissolved readily in strong reducing acids and, when powdered, in weak acids if organic material is present. Table I and Text-fig. 1 show that manganese dioxide deposited in the lung capillaries by intravenous injection begins to diminish in amount within 3 hours after injection, that there is simultaneous increase of manganese in the liver, and that within 6 hours after injection the total amount contained by these organs has fallen when compared with the amount which must have been present immediately after injection. We have performed a number of experiments in an attempt to discover whether the manganese dioxide deposited originally on the inner surface of the endothelium of the lung capillaries is washed away by the blood current and is then caught more permanently by the Kupffer cells lining the liver capillaries, or whether the manganese dioxide is phagocytosed in the lung, broken down into soluble manganese, returned to the blood stream, and removed from the blood by the liver. In regard to the first of these alternatives, it will be noted that the transfer of manganese from lungs to liver begins promptly after injection and takes place with considerable rapidity. Fenn (5) has recently shown that manganese dioxide is taken by phagocytes with the greatest readiness. His data, however, give no idea as to how firmly the substance is held after being phagocytosed. In the rabbit, in which practically the entire injection of manganese dioxide is removed from the blood stream by the Kupffer cells of the liver, we have found both dissolved and insoluble manganese in the bile collected following the injection. It is true that the amount of manganese dioxide which reaches the bile in these animals is never great, but the fact that it appears at all shows that phagocytosed manganese dioxide can escape solution for a considerable period of time.

In regard to the second alternative, namely the solution of manganese in the lungs with subsequent removal of dissolved manganese

from the blood by the liver, a number of points arise. First, the manganese dioxide employed is in very fine subdivision, a state most favorable for immediate reduction. It is, furthermore, both in the lungs and in the liver, deposited on endothelium which, in great part at least, is bathed by highly venous blood. Jacobs (6) has shown that intracellular acidity due to carbonic acid may be far greater than the acidity of the surrounding medium. This is due to the high penetrating power of the carbonic acid molecule as compared with that of other acids. If these facts are taken into consideration, it is evident that in the lungs as well as in the liver the surroundings are very favorable for the reduction of phagocytosed manganese dioxide particles, the conversion of the manganese to carbonate, and the diffusion of this carbonate into the tissue fluids or back into the blood stream from which it will subsequently be removed by the liver. In this general connection, it is worth noting that Gardner and Dworski (7) have recently shown that marble dust inhaled by guinea pigs is removed by solution in the body fluids and also apparently by solution after phagocytosis. That calcium carbonate should be so handled is not surprising. Minot[3] has recently found that lead chromate insufflated into the lungs of cats is removed from the lungs apparently after being dissolved. This substance is quite insoluble, and the fact that it is broken down within the lungs is evidence of the ability of the lung phagocytes to attack foreign substances.

In Section II of this paper it has been shown that rhodonite deposited in the lungs of cats apparently does not undergo transfer to the liver before excretion; that is to say, as manganese disappears from the lungs there is no corresponding rise in the amount of this substance recovered from the liver (Text-fig. 3). The mechanism of primary removal of rhodonite from the blood by the lungs is undoubtedly similar to that operative with manganese dioxide, but appreciable amounts of the silicate do not shift from the lungs to the liver either in soluble or in insoluble form. This fact, however, has no necessary bearing on the mechanism of removal of manganese dioxide from the lungs. With the silicate, retention of particles in the lungs may depend upon many factors, such as the penetrating power of

[3] Minot, A. S., unpublished data.

the sharp silica particles, a greater retentive power of phagocytes for these particles once they are ingested, and other possibilities which need not be mentioned.

Table II indicates the rapidity with which the rabbit gets rid of manganese dioxide. In this case the whole process is carried out by the liver. The initial deposition of the substance on the Kupffer cells is readily seen microscopically, and occasional particles can be found in the liver parenchyma. Careful chemical examination of many specimens of bile from injected rabbits has shown that not only is dissolved manganese excreted in this way but also intact particles of manganese dioxide pass into the bile. In a series of four rabbits in which the common bile duct was cannulated and bile collected for periods of 8 to 15 hours after injection of average doses of approximately 15 mg. of manganese dioxide, the bile obtained was centrifuged for 50 to 60 minutes at high speed, and the supernatant liquids and the sediments obtained were analyzed for manganese. The average percentages of the total manganese recovered in these biles were 12.2 per cent in the supernatant liquids and 87.8 per cent in the sediments.[4] This clearly indicates that manganese dioxide is excreted in the bile in particulate as well as in soluble form.

That dissolved manganese is excreted in the bile has been known for a long time (8), but that particles of any sort pass through the liver is, we believe, a unique observation. In contrast to these inorganic substances, it is noteworthy that typhoid bacilli, given intravenously to rabbits, reach the bile within the gall bladder from the blood and apparently do not pass through the liver (9). It is possible, however, that injected organisms may pass into the liver on their way to the bile but be killed in the process so that cultures made from hepatic duct bile would be sterile.

Dissolved manganese is excreted not only by way of the bile but also directly by the blood into the intestines. A negligible amount of dissolved manganese reaches the urine, and it seems possible that

[4] Because of its variability the normal manganese content of bile has not been subtracted from the recoveries upon which these figures are based. As a result, the percentages of manganese found in the supernatant liquids are doubtless somewhat higher than they would be had the injected manganese only been considered.

a few particles of manganese dioxide which reach the alveoli may pass up the trachea, in which case the manganese would be swallowed and would eventually be excreted in the feces. In the case of rhodonite, we have gained definite evidence that the particles deposited in the lungs do in some measure take this last route of excretion, but for manganese dioxide we have not obtained convincing results on this point.

The experiments with manganese meta-silicate include, first, a small group upon the effect of particle size on the immediate site of deposition in cats. Text-fig. 2 presents a graphic summary of these experiments. In a previous paper, it has been shown that neither circulatory conditions in the animal, nor, within reasonable limits, the volume of the suspension injected, affects the immediate distribution of the suspended manganese dioxide. This chart shows the greater ease with which larger particles are arrested in the lungs. Miller (10) has shown that the blood capillaries of the mammalian lung average about 7μ in diameter. The largest particles in the suspensions used in the present work were 1.3μ in diameter and microscopic examination of lung specimens following injections of the material have never shown emboli. We are disinclined to believe that the difference observed in the deposition of the three sizes of particles is due to capillary blockage. The chances that the largest particles will collide with the capillary walls and be held are, of course, greater than with the smaller ones. This has been brought out by Fenn (4) in his studies on phagocytosis of carbon and silica, and he has also shown that large particles are more readily seized and held than small ones (5). It is of interest that as our particles reach colloidal dimensions the number garnered by the liver increases.

In rabbits injected with particles of varying size and with true solutions of manganese, practically the entire injection may be recovered from the liver.

Voigt (11), in the case of colloidal silver, Duhamel (12), employing colloidal solutions of silver, platinum, selenium, mercury, copper, and iron, and Sabbatani and Salvioli (13), using colloidal ferric oxide, have noted the same thing. The last authors have been interested entirely in initial deposition. Gye and Purdy (14) have made repeated intravenous injections of colloidal silica in rabbits and have observed the appearance of fibrosis in the liver, spleen, and kidneys. They

regard these pathological developments as expressions of an inherent toxic property of the silica sol employed. Their work is not controlled by injections of any other colloidal material, and it is obvious that the sites of fibrosis observed by them—liver, spleen, and kidneys—are those chosen for deposition from the blood of all the different substances so far studied in which the individual particles measure from colloidal up to 2μ in size. We have not as yet made experiments with repeated injections of manganese compounds but have observed increased fibrosis in the liver of cats receiving single large injections of rhodonite with a maximum particle size of 1.3μ. Gye and Purdy (14) attribute the fibrosis observed by them to the presence of silica sol, which they believe to be a capillary poison. In a third paper by Gye and Kettle (15), this idea is summarized as follows:

"Our work has been based on the known facts—(1) that colloidal silica is a cell poison; (2) that colloidal silica is the most easily formed soluble form of silica, the circulation of silica in nature depending upon this; and (3) that living matter (soil bacteria) is able to break up mineral silicates with formation of soluble silica (Bassalik, 1913). We judged it probable that the fibrosis brought about by finely-divided silica was due to the *slow* formation in the tissues of silica sol, either directly or through the intermediate formation of sodium silicate which is decomposed by carbonic acid, the soil formed acting as a cell poison. The direct proof of this has been found impossible, and we are, therefore, compelled to fall back upon indirect evidence."

The evidence presented consists of a series of subcutaneous injections of orthosilicic acid, so called soluble silica—in reality, a colloidal solution of silica—and insoluble silica in the form of silicon dioxide. The latter substance is quartz and is not a silicate, so that the reasoning used by Gye and Kettle does not necessarily apply to it. When these substances were injected they induced similar lesions.

"The only obvious difference is that the reaction is more rapid and more transient with colloidal silica, and our findings support very strongly the view that SiO_2 becomes soluble by hydration in the tissue; but, inasmuch as with insoluble silica the reaction is somewhat delayed, the lesions produced by this substance lend themselves rather more readily to analysis than those produced by colloidal silica."

It is difficult to conceive that SiO_2 can be rendered soluble in the manner assumed by these authors, and even if this is the case it cannot as yet be considered that the fibrotic changes observed by Gye and Purdy in the liver of rabbits, following intravenous injection of colloidal silica, are certainly the expression of a specific toxic property of silica and are not foreign body reactions induced by a substance which possesses an extraordinary quality of permanence after deposition.

Table V and Text-fig. 3 show that rhodonite suspensions injected intravenously into the cat do remain in the animal for a long period and that there is no shift from lungs to liver as with manganese dioxide. On microscopic examination of the liver of these animals

one finds, after several weeks, evidences of fibrous tissue increase. This cannot be made out with definiteness in the lungs, but we believe that it will appear on repeated injection, and experiments of this type are now in progress. Rhodonite is, therefore, a suitable substance with which to attempt the studies on lung fibrosis originally projected, and, with it as a standard injection, one should be able to compare the effects of carbon, cement, jute, and other dusts which cannot be quantitated chemically but which have been suspected, both directly and indirectly, of causing lung damage.

As with manganese dioxide, we have attempted to find out how rhodonite leaves the body. In the cat evidence has been gained of a constant slow removal from the lungs *via* the trachea; that is, particles deposited on the inside of the blood capillaries pass into the alveoli and eventually reach the trachea. In regard to the rabbit, Table VI shows the steady elimination which occurs from the liver. As with the dioxide, the bile obtained after the injection of rhodonite contained both dissolved and particulate manganese. The fact that rhodonite, a silicate, is dissolved in the body makes us turn once more to the ideas of Gye and his coworkers. Undoubtedly, at least a fraction of this silicate is broken down, and one of the products may possibly be colloidal silica. The actual establishment of this supposition as a fact would appear impossible, and it is impossible as yet to decide what fraction of the silicate is eliminated intact and what fraction after disintegration.

SUMMARY.

1. Suspensions of manganese dioxide given intravenously to the cat are virtually eliminated from the body in 1 week (168 hours).

2. As this process of removal goes on, there is a transfer of manganese from lungs to liver.

3. Similar suspensions of manganese dioxide given intravenously to rabbits are excreted more rapidly, owing to the fact that in these animals the lungs are not involved and the whole process of removal from the blood is carried on by the liver.

4. A method for the quantitation of rhodonite (manganese metasilicate) is given.

5. Three different suspensions of rhodonite, varying in the size of the largest particles from 1.3 to 0.2μ, when injected intravenously

into cats show a greater and greater tendency toward arrest in the liver as the size of the individual particles becomes smaller.

6. When rhodonite suspensions containing particles averaging 1.1μ in diameter are injected intravenously into cats, the primary sites of deposition are similar to those observed for manganese dioxide suspensions similarly injected. The disappearance of rhodonite is exceedingly slow and is apparently completed in about 50 days following injection.

7. Rhodonite should prove a suitable substance with which to attempt the production of lung fibrosis in cats through the medium of intravenous injection. Manganese dioxide, while a useful agent for studying the immediate sites of localization of particulate material carried by the blood, lacks permanency after deposition and is consequently unsuitable for studies upon chronic changes induced by foreign substances.

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ARSENICAL COMPOUNDS IN THE TREATMENT OF BLACKHEAD IN TURKEYS.

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In a previous investigation[1] an attempt was made to prevent blackhead infection in turkeys by the addition of an amebicide, *Chaparro amargosa*, to their daily ration. While this procedure resulted in an almost complete disappearance of *Entamœba gallinarum*, a non-pathogenic ameba commonly found in the ceca of the turkey, it failed to prevent blackhead infection.

A study of the blackhead parasite, at this time, furnished morphological evidence indicating that it is a flagellate rather than a true ameba.[2,3]

The discovery that blackhead may be produced experimentally in young turkeys by feeding them large numbers of the ripe eggs of *Heterakis papillosa*, the common intestinal worm of poultry,[4] and also the demonstration that a fatal form of the disease may be produced in turkeys by the inoculation of active blackhead lesions,[5] have furnished reliable experimental methods of producing the infection, which may be utilized for therapeutic tests. In the course of successive seasons various drugs generally accepted as specifics in the treatment of protozoan diseases in man have been tested.

Tartar emetic, of recognized value in leishmaniasis, a flagellate infection, was injected intravenously at intervals, in toxic doses, into a large turkey inoculated with blackhead, but it failed to prevent the development of the disease or its fatal termination.

Large doses of quinine hydrochloride injected either into the veins or deep in the breast muscle also failed to check the course of the inoculated disease.

Emetine hydrochloride given subcutaneously in large doses at 24 and 48 hour intervals failed to prevent the development of the inoculated disease, but of two treated turkeys one eventually recovered.[6] No special significance was attached to this result, for one recovery had already been noted in a series of untreated cases of the inoculated disease.

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[1] Tyzzer, E. E., *J. Med. Research*, 1919–20, xli, 211.

[2] Tyzzer, E. E., *J. Med. Research*, 1919, xl, 1.

[3] Tyzzer, E. E., *J. Parasitol.*, 1920, vi, 124.

[4] Graybill, H. W., and Smith, T., *J. Exp. Med.*, 1920, xxxi, 647.

[5] Tyzzer, E. E., and Fabyan, M., *J. Infect. Dis.*, 1920, xxvii, 207.

[6] Tyzzer, E. E., Fabyan, M., and Foot, N. C., *J. Infect. Dis.*, 1921, xxix, 268.

Subsequently Wegeforth and Wegeforth[7] reported success in both the prevention and the cure of natural blackhead in turkeys by the addition of ipecac to the daily ration. On account of the widespread interest in this report and since it is possible to be misled with respect to both the incidence of the disease and the outcome of natural blackhead in a flock, it seemed important to subject this drug to experimental test. We found that ipecac could be administered to young turkeys in amounts considerably larger than those used by the above investigators without any untoward effect.[8] A group of twenty-three turkeys was furnished with infectious material in the form of hen yard dirt mixed with dry mash. Eleven of these received ipecac daily, while the others served as controls. Ipecac in the large doses employed failed wholly to prevent blackhead, but appeared to have some effect, in that the onset of the disease was delayed in the treated birds. None of the eleven turkeys to which the ipecac was administered escaped infection, and the only one of these to recover, failed to develop normally. From these results, it did not appear that ipecac should be accepted as a specific for blackhead.

Precipitated sulfur added in large amounts to the food seemed also to retard slightly the onset of blackhead.[8] As sulfur is relatively inert and does not prevent the development of either intestinal worms or the protozoon of blackhead, it is probable that any benefit obtained from either ipecac or sulfur is due to its evacuant action.

Spontaneous Blackhead.

During the present season (1922) fifteen broods totalling 154 turkeys were hatched between May 6 and August 26. All were hatched in an incubator, from which they were transferred to a brooder, and about a week later were placed out of doors in cages each furnished with a warm hover. The broods of approximate ages were subsequently united into three flocks, each of which was provided with a yard and shelter.

The first case of blackhead appeared on June 8, and new cases continued to develop with considerable frequency up to the end of October, but only three cases occurred in the 2 following months. Since these turkeys were being raised primarily for market purposes, the continued appearance of new cases of infection together with the high mortality was not promising. The disease was so generally distributed throughout the various broods that to move them to a

[7] Wegeforth, H. M., and Wegeforth, P., *J. Pharmacol. and Exp. Therap.*, 1921, xvii, 249.

[8] Tyzzer, E. E., and Fabyan, M., *J. Exp. Med.*, 1922, xxxv, 791.

new range, while it might lower the incidence of the disease, would presumably contaminate more ground. Most of the following observations were made in the course of attempts to cure turkeys naturally infected with blackhead. This preliminary work served to indicate the drugs which were most effective, and towards the end of the season an experiment was carried out for the purpose of putting the more promising ones to a rigid experimental test in the treatment of inoculated blackhead.

Throughout the summer any turkey showing symptoms of the disease was placed in a special well grassed yard, the "hospital," in order to avoid its being abused and possibly killed by the healthy turkeys. Spontaneous recovery occurred early in the season in one case diagnosed as blackhead, but subsequently through June and July each case terminated fatally. Late in the season a 7 pound male bird, after showing mild symptoms for several days, also recovered without treatment. There were therefore only two recoveries in sixteen untreated cases of the disease, a mortality of 87.5 per cent.

Neoarsphenamine Treatment.—Beginning early in August neoarsphenamine (sodium 3,3′-diamino-4,4′-dihydroxy-arseno-benzene-*N*-methylene sulfinate) was injected into each case as soon as possible after the appearance of characteristic symptoms. Both American and French products were employed. The powder was dissolved in a small amount of sterile distilled water and the concentrated solution injected at once into the wing vein. It was evident that this solution caused severe pain when introduced into fixed tissues. Injection into the breast muscle produced extensive local edema which evidently hastened the death of young birds. Large doses were at first employed, in some instances as much as ten times the maximum human dose in proportion to the body weight being used. The immediate mortality was such that the dosage was lowered and from three to five times the maximum human dose, *i.e.* from 0.04 to 0.06 gm. per kilo, was given. While healthy turkeys are not seriously affected by these doses, sick birds show a pronounced prostration which appears in from 10 to 20 minutes and may last 6 hours or longer.

The young turkey is evidently much more susceptible than the rat to neoarsphenamine, for Federal Government regulations[9] require

[9] Roth, G. B., *Pub. Health Rep., U. S. P. H.*, 1921, xxxvi, 2523.

that white rats weighing from 100 to 150 gm. shall survive 7 days after the intravenous injection of 0.2 gm. per kilo, or from three to five times the doses here employed in the treatment of spontaneous blackhead. The smaller turkeys often succumb within 24 hours, but nearly all those surviving the treatment show a pronounced improvement on the following day. In infected turkeys the toxic reaction of neoarsphenamine is almost immediate, and the delayed effects noted in rats[10] have not been observed. As in rats, there is a pronounced tendency to hemorrhage following injection, so that unless pressure is maintained for a considerable time over the punctured wing vein, large hematomas result. One turkey acted as though blind following the injection, but after a few days showed improvement in vision. On further observation the difficulty appeared to be one of accommodation rather than actual blindness, in that distant objects appeared near. Many months after recovery from the blackhead infection this turkey still showed slightly defective accommodation and usually pecked twice in order to strike a particle of food.

There are two features which occur quite constantly in spontaneous blackhead that of themselves militate against recovery. First, the infected turkeys do not rest normally by squatting on the ground, but keep constantly on their feet, either moving listlessly about or standing hunched up for long periods usually with head under wing. Secondly, there is loss of appetite, which, with the diarrhea usually present, results in weakness and a progressive loss of weight.

As the toxic effects of the neoarsphenamine disappear, the treated turkeys show at first a pronounced thirst, drinking large amounts of water, and then an improvement in appetite. They subsequently rest for long periods, squatting normally on the ground. Such improvement in function can scarcely fail to have a favorable influence on the course of the disease. The treated turkeys soon become more brisk in their movements, and it may be difficult after a few days to distinguish them from the healthy birds. The subsequent course of the disease varies in different cases; some show steady improvement and are fully recovered within a week, others are alternately active and depressed, either recovering after a prolonged convalescence or

[10] Roth, G. B., *Pub. Health Rep., U. S. P. H.*, 1921, xxxvi, 1990.

eventually succumbing to the disease. The most favorable response to treatment was obtained in the early stages of infection, and recovery was rarely obtained in advanced cases. One turkey (No. 15) had a relapse with characteristic symptoms following a period of marked improvement. The results of the injection of neoarsphenamine in cases of blackhead are furnished in Table I. Although the reduction of the mortality to 41 per cent was encouraging, the high cost of this drug, its instability, the difficulty of obtaining ampules in amounts suitable for given occasions, and its high toxicity in doses sufficiently large to influence the disease constitute serious obstacles to its employment.

Arsenious Acid Treatment.—It was thought possible that small amounts of arsenious acid added to the daily ration might serve to prevent infection. On September 7, liquor acidi arsenosi, an acidulated 1 per cent aqueous solution of arsenic (As_2O_3), was added to the daily ration of sour milk in an amount to furnish 1 cc. to 20 kilos of body weight of turkeys. Thus in proportion to the body weight about one-tenth of the maximum sublethal dose for a rabbit was given each day. On September 11, the above dose was doubled, on September 12, it was tripled, and again increased on September 16, when about one-half the sublethal rabbit dose was given. On account of the death at this time of a large turkey without any lesions of blackhead, no arsenic was given on or after September 17. For the 8 days preceding the administration of arsenic there had been on the average approximately one new case of blackhead a day. This rate of incidence was somewhat lower for a time after the treatment was established but showed a pronounced increase following the increase in dosage of arsenic. It was obvious that the administration of this drug failed to prevent blackhead, and it is even possible that the larger doses finally given were increasing the incidence of infection. It is of interest to note that one turkey weighing nearly 3 kilos evidently succumbed to arsenical poisoning. The symptoms were almost identical with those of blackhead infection; *i.e.*, weakness, loss of appetite, and chrome-yellow droppings. There was, however, no marked loss of weight. On postmortem examination the liver and spleen were engorged and of a dark purplish color. There were no blackhead lesions either in the ceca or in the liver.

TABLE I.

Group.	Turkey No.	Date of onset of disease.	Symptoms.	Injections.	Result.	Remarks.
		1922				
	1	June 8	Typical.		June 13. D.	Typical blackhead lesions.
	2	" 15	Mild.		Recovered.	Recovery from a mild case of blackhead.
	3	" 17	Typical.		June 25. D.	Typical blackhead lesions.
	4	" 26	"		" 28. "	" " "
	5	July 6	"		July 10. "	" " "
1 Untreated.	6	" 10	"		" 21. "	" " "
	7	" 12	"		" 24. "	" " "
	8	" 14	"		" 21. "	" " "
	9	" 20	Severe.		" 20. "	Cecal blackhead; no liver lesions.
	10	" 25	Typical.		" 25. K.	Blackhead. Recovery precluded by the severity of the infection.
	11	Aug. 1	"		Aug. 8. D.	Typical blackhead lesions.
	12	" 2	"		" 8. "	" " "

1 Untreated.	31	Sept. 7	Mild.		Recovered.	A spontaneous recovery from probable blackhead.
	33	" 13	Typical.		Sept. 16. D.	Typical blackhead lesions.
	36	" 18	"		" 23. "	" " "
	63	Oct. 26	"		Oct. 28. K.	Blackhead. Recovery unlikely with such extensive liver involvement.
2 Treated with neo-arsphenamine.	13	Aug. 7	Typical.	Aug. 9. 0.10 gm. in vein. " 12. 0.10 " " "	Recovered.	First injection followed by impairment of vision which gradually improved but remained defective.
	14	" 10	"	" 10. 0.10 " " " " 12. 0.15 " " "	"	Normal 14 days after onset.
	15	" 10	"	" 10. 0.05 " " " " 12. 0.05 " " " " 22. 0.09 " " "	"	Marked improvement after second injection followed by relapse on Aug. 21. Apparent recovery followed the third injection. On Sept. 10, again very ill; symptoms atypical; photophobia; apparently in pain. Recovery without further treatment.
	16	" 10	"	" 10. 0.08 " " " " 12. 0.010 " " "	"	Normal 14 days after onset.

TABLE I—*Continued.*

Group.	Turkey No.	Date of onset of disease.	Symptoms.	Injections.	Result.	Remarks.
		1922				
	17	Aug. 10	Typical.	Aug. 10. 0.07 gm. in vein and breast.	Aug. 11. D.	Dose of neoarsphenamine undoubtedly lethal. Hemorrhage into peritoneum and ceca. Typical blackhead lesions.
	18	" 11	"	" 12. 0.05 " " "	" 13. "	Lethal dose of neoarsphenamine. Early blackhead lesions.
	19	" 11	"	" 12. 0.05 " " "	" 12. "	Lethal dose of neoarsphenamine. Early blackhead lesions.
2 Treated with neoarsphenamine.	20	" 11	Severe.	" 12. 0.05 " " " " 22. 0.05 " " " " 25. 0.05 " " "	Recovered.	Slow recovery.
	21	" 14	Typical.	" 18. 0.10 " " " " 22. 0.15 " " "	"	Improvement gradual.
	22	" 15	Severe.	" 18. 0.05 " " "	Aug. 19. D.	Typical blackhead lesions.
	23	" 23	Typical.	" 25. 0.075 " " " " 30. 0.06 " " "	" 31. "	" " "
	24	" 30	"	" 30. 0.06 " " " Sept. 2. 0.075 " " " " 6. 0.06 " " "	Recovered.	Rapid improvement after last injection.

	25	Aug.	30	Typical.	Aug.	30.	0.02	gm.	in	vein.	Sept.	7.	D.	Typical blackhead lesions.
					Sept.	2.	0.025	"	"	"				
					"	6.	0.03	"	"	"				
	26	Sept.	1	Severe, acute.	"	2.	0.20	"	"	"	"	2.	"	An excess of neoarsphenamine. Early blackhead lesions.
	27	"	1	Typical.	"	2.	0.15	"	"	"	"	15.	"	Blackhead lesions; healing.
					"	6.	0.12	"	"	"				
					"	13.	0.075	"	"	"				
	28	"	5	"	"	6.	0.13	"	"	"	Recovered.			Severe infection in a large turkey. Recovery slow; 2½ wks.
2 Treated with neo-arsphenamine.					"	13.	0.075	"	"	"				
	29	"	6	"	"	6.	0.07	"	"	"	"			Steady improvement following injection.
					"	8.	0.05	"	"	"				
	30	"	6	"	"	6.	0.04	"	"	"	Sept.	8.	D.	Lethal dose of neoarsphenamine. Typical blackhead lesions.
					"	8.	0.05	"	"	"				
	32	"	10	Mild.	"	21.	0.10	"	"	"	Recovered.			At no time very ill.
	37	"	21	Typical.	"	21.	0.07	"	"	"	"			Rapid improvement.
	38	"	21	"	"	21.	0.05	"	"	"	"			Gradual "
	39	"	21	"	"	21.	0.08	"	"	"	"			" "

TABLE I—*Continued.*

Group.	Turkey No.	Date of onset of disease.	Symptoms.	Injections.	Result.	Remarks.
		1922				
	40	Sept. 21	Typical.	Sept. 21. 0.5 cc. of 1 per cent solution in breast.	Sept. 24. D.	Typical blackhead lesions.
	41	" 21	"	Sept. 21. 0.5 cc. of 1 per cent solution in breast. Sept. 24. 2 cc. of 1 per cent solution in breast. Sept. 29. 1.5 cc. of 1 per cent solution in vein.	" 29. "	" " "
	42	" 21	"	Sept. 21. 0.5 cc. of 1 per cent solution in breast. Sept. 24. 2 cc. of 1 per cent solution in vein.	Recovered.	Appears well 8 days after onset.
3 Treated with atoxyl.	48	" 24	"	Sept. 24. 5 cc. of 1 per cent solution in vein. Sept. 29. 3.5 cc. of 1 per cent solution in vein.	Sept. 30. D.	Typical blackhead lesions.
	49	" 27	"	Sept. 27. 5 cc. of 1 per cent solution in vein. Sept. 29. 5.5 cc. of 1 per cent solution in vein.	Recovered.	Extreme emaciation; weakness after many weeks.
	50	" 27	"	Sept. 27. 2 cc. of 1 per cent solution in vein.	"	Recovery slow.
	51	" 29	"	Sept. 29. 5 cc. of 1 per cent solution in vein.	Sept. 29. D.	Typical blackhead lesions.

	34	Sept. 13	Typical.	Sept. 13. 0.10 gm. of neoarsphenamine in vein. Sept. 29. 5 cc. of 1 per cent solution of atoxyl in vein.	Oct. 1. D.	Typical blackhead lesions. Largest male.
	35	" 13	"	Sept. 13. 0.10 gm. of neoarsphenamine in vein. Sept. 21. 0.14 gm. of neoarsphenamine in vein. Sept. 29. 4 cc. of 1 per cent solution of atoxyl in vein.	Recovered.	Disease ran a chronic course. Large hen turkey.
4 Treated with atoxyl and neoarsphenamine.	44	" 21	"	Sept. 21. 0.05 gm. of neoarsphenamine in vein. Sept. 29. 3 cc. of 1 per cent solution of atoxyl in vein.	Sept. 30. D.	Typical blackhead lesions.
	45	" 21	Severe.	Sept. 21. 0.05 gm. of neoarsphenamine in vein. Sept. 29. 3 cc. of 1 per cent solution of atoxyl in vein.	" 30. "	" " "
	46	" 21	"	Sept. 21. 0.13 gm. of neoarsphenamine in vein. Sept. 29. 4 cc. of 1 per cent solution of atoxyl in vein.	Recovered.	Disease ran chronic course.
	47	" 21	"	Sept. 21. 0.15 gm. of neoarsphenamine in vein. Sept. 29. 3 cc. of 1 per cent solution of atoxyl in vein.	"	" " " "

TABLE I—*Continued.*

Group.	Turkey No.	Date of onset of disease.	Symptoms.	Injections.	Result.	Remarks.
		1922				
	52	Oct. 7	Typical.	Oct. 9. 7 cc. of 25 per cent solution in vein.	Recovered.	Prompt improvement. Well 7 days after onset.
	53	" 8	"	Oct. 9. 8 cc. of 25 per cent solution in vein. Oct. 15. 12 cc. of 25 per cent solution in vein.	"	Improvement after second injection.
	54	" 8	"	Oct. 9. 6 cc. of 25 per cent solution in vein. Oct. 15. 6 cc. of 25 per cent solution in vein.	"	Improvement gradual.
5 Treated with tryparsamide.	55	" 10	Severe.	Oct. 10. 4 cc. of 25 per cent solution in vein. Oct. 12. 4 cc. of 25 per cent solution in vein. Oct. 15. 5 cc. of 25 per cent solution in vein.	Oct. 16. D.	Liver extensively involved. Difficulty in finding food on account of pock lesions on eyelids.
	56	" 15	Typical.	Oct. 15. 6 cc. of 25 per cent solution in vein. Oct. 20. 4 cc. of 25 per cent solution in vein. Oct. 26. 6 cc. of 25 per cent solution in vein.	Recovered.	Improvement gradual.

5 Treated with tryparsamide.	57	Oct. 15	Typical.	Oct. 16. 15 cc. of 25 per cent solution in vein.	Recovered.	Improvement prompt.
	58	" 16	"	Oct. 16. 5 cc. of 25 per cent solution in vein and 22 cc. subcutaneously. Oct. 26. 14 cc. of 25 per cent solution in vein.	"	" gradual.
	59	" 17	"	Oct. 17. 26 cc. of 25 per cent solution in vein. Oct. 18. 11 cc. of 25 per cent solution subcutaneously.	"	" prompt.
	60	" 18	"	Oct. 18. 9 cc. of 25 per cent solution in vein.	"	" "
	61	" 18	"	Oct. 18. 7 cc. of 25 per cent solution in vein.	"	" "
	62	" 18	Severe.	Oct. 20. 8 cc. of 25 per cent solution in vein.	Oct. 26. D.	Typical blackhead lesions.
	64	" 29	Typical.	Oct. 29. 22 cc. of 25 per cent solution in vein.	Recovered.	Improved steadily.
	65	" 29	"	Oct. 31. 14 cc. of 25 per cent solution in vein.	"	" "

TABLE I—*Concluded.*

Group.	Turkey No.	Date of onset of disease.	Symptoms.	Injections.	Result.	Remarks.
		1922				
	66	Oct. 30	Typical.	Oct. 31. 7 cc. of 25 per cent solution in vein and 8 cc. subcutaneously.	Recovered.	Improved steadily.
	67	Nov. 24	"	Nov. 25. 14 cc. of 25 per cent solution in vein.	"	" "
5 Treated with tryparsamide.	68	" 25	"	Nov. 25. 8 cc. of 25 per cent solution in vein and 8 cc. subcutaneously.	"	" "
	69	Dec. 17	"	Dec. 17. 14 cc. of 25 per cent solution in vein.	"	" "
6 Treated with atoxyl and tryparsamide.	43	Sept. 21	Typical.	Sept. 21. 0.5 cc. of 1 per cent solution of atoxyl in vein. Oct. 14. 4 cc. of 25 per cent solution of tryparsamide in vein.	Recovered.	Course of infection chronic.

While it is possible that the above estimated doses of arsenic may not be especially harmful to turkeys, bad results may follow the administration of so powerful a poison in the general ration for a flock. One especially greedy bird may take much more than his share of the food, and if the arsenic is given in milk or water the thirst produced by the poison may result in its being taken in undue amount.

To obtain an idea of the amount of arsenic that may be administered in a single dose, two turkeys, one weighing 1½ and the other 2 kilos, were given 0.7 and 1 cc. respectively of liquor acidi arsenosi by mouth. The first had 2 days previously received 0.05 gm. of neoarsphenamine and the second 11 days later received 0.08 gm. of the same, in both cases injected intravenously. These two turkeys were markedly prostrated after taking the arsenic, one not eating for several days. There was eventually some improvement, but both finally died without recovery from the blackhead infection. Since both birds were weakened by the disease and received additional arsenic in the neoarsphenamine injected on another occasion, it appears probable that the turkey may withstand as large doses of arsenic as the rabbit. The results obtained in these two instances also show that arsenic when given in large doses to infected turkeys fails to control blackhead.

Atoxyl Treatment.—Atoxyl was employed in a small number of cases, but as the turkeys had previously been receiving arsenic in their food, it was not considered advisable to employ more than very moderate doses. Consequently the results are not especially conclusive. There were, however, several recoveries among the treated birds.

Tryparsamide Treatment.—Another arsenic compound, tryparsamide, or *N*-phenylglycineamide-*p*-arsonic acid, prepared by Jacobs and Heidelberger[11] and introduced by Brown and Pearce[12–17] in the treatment of trypanosome and spirochete infections was finally utilized

[11] Jacobs, W. A., and Heidelberger, M., *J. Exp. Med.*, 1919, xxx, 411.
[12] Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1919, xxx, 417.
[13] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1919, xxx, 437.
[14] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1919, xxx, 455.
[15] Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1919, xxx, 483.
[16] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1921, xxxiii, 193.
[17] Pearce, L., *J. Exp. Med.*, 1921, xxxiv, No. 6, Suppl. 1.

in the treatment of turkeys infected with blackhead. A 25 per cent solution of tryparsamide in distilled water was injected, usually into the wing vein but occasionally subcutaneously. Since this solution on boiling became yellowish in the course of a few days, the salt was as a matter of routine dissolved in sterile distilled water and used without further sterilization. It was thought inadvisable to use the dregs of the solution, which showed a fine sediment, for intravenous injection, and this portion was therefore injected subcutaneously. In contrast with neoarsphenamine, tryparsamide produced no evidence of pain when injected subcutaneously, and no more than a transient local edema followed the injection of large doses beneath the skin on the back of the head and neck. Little or no bleeding resulted from the puncture of the vein. No blindness or other untoward result followed the tryparsamide treatment of cases of natural infection. After the intravenous injection of the drug there was a variable amount of weakness which lasted only a few hours at most, and this was usually followed by pronounced improvement. The birds at first showed a marked thirst, after which the appetite returned. Another favorable effect was that they would now squat down and rest.

From a study of the pathology of this disease, it is readily understood why recovery occurs so infrequently in young turkeys if the disease is allowed to run its natural course. Not only is the function of the cecum more or less completely interfered with, but often the destruction of liver parenchyma is so extensive as to preclude recovery. In a series of seventeen cases of spontaneous blackhead treated with tryparsamide there were two deaths, or a mortality of less than 12 per cent.

One of the two tryparsamide-treated birds that failed to recover had both eyes closed with lesions of bird-pox so that it was unable to feed without assistance, the other was extremely ill before receiving treatment. Stained sections of the diseased liver of the first of these showed a total disappearance of liver parenchyma in the involved areas, leaving a reticulum transversed by blood vessels and in certain areas extensively infiltrated with cells, chiefly of lymphoid type. There were early regenerative changes present but repair was far from complete.

The dosage employed was based on that determined by Brown and Pearce[12] for laboratory animals. From 1 to 1.5 gm. per kilo may be safely injected intravenously into infected turkeys, but somewhat smaller doses should be employed with very young turkeys. From the favorable results here obtained, it should not be assumed that tryparsamide is a certain cure for all cases of blackhead. On account of the extensive destruction of the liver parenchyma, it would appear futile to undertake the cure of blackhead in an advanced stage. The treatment should begin as soon as the diagnosis can be established. Experience has shown that delay in beginning treatment both reduces the probability of recovery and prolongs the period of convalescence if the bird survives. Since blackhead may produce death within a relatively short period after the lesions are sufficiently developed to produce symptoms, prompt action is more essential than in the treatment of a mild or more chronic disease. After a time the following course was adopted for the detection of cases of blackhead at the earliest possible moment. The ground beneath the night shelter was examined each morning for sulfur droppings and, if they were found, a search was made for a moping turkey, which was then placed under observation until the diagnosis could be established by the discharge of sulfur droppings, lack of appetite, etc., and by the elimination of other possible ailments such as obstruction of the crop or other portions of the alimentary tract. If the symptoms were characteristic of blackhead, the turkey was placed in the hospital yard and treatment at once instituted.

From the results obtained in the treatment of spontaneous blackhead, it appeared that tryparsamide was the most effective of the compounds employed. The percentage of recoveries following the treatments outlined above should not, however, be taken as a true index of the therapeutic value of the drugs. It is probable that the mortality in cases of blackhead would have diminished as the season advanced, and it seems fairly well established that the chances of recovery increase as the turkey matures. The turkeys of the group treated with tryparsamide were on the average somewhat larger than those of the other groups, but they were of various ages and sizes. Furthermore, recovery followed tryparsamide treatment in a number of relatively small turkeys weighing from 1 to 2 kilos (roughly 8 to 15 weeks old).

The clinical action of the drugs with respect to the amelioration of serious symptoms should also be considered. Neoarsphenamine in adequate doses for the treatment of blackhead shows a pronounced toxicity and may give an immediate high mortality in young birds. Improvement of appetite, increase of vigor, and ability to rest usually succeed the period of prostration that follows the injection of this drug. Tryparsamide, however, may be given in large doses without producing marked prostration and, judging from clinical symptoms, acts fully as beneficially on the course of the disease.

On account of the lack of adequate controls with respect to age, season, and severity of infection in the treatment of spontaneous cases of blackhead, it seemed desirable to subject these drugs to experimental test.

Inoculated Blackhead.

The following experiment was planned to compare the effects of treatment of inoculated blackhead in turkeys of approximate age and weight with these drugs. Inoculated blackhead has been found to run a more uniform course than the natural infection, and since it is possible to make direct observations on the development of the local lesion, all question of error in diagnosis is eliminated.

Experiment.—October 9, 1922. Twelve young turkeys weighing from 555 to 890 gm. were inoculated in the left breast with bits of liver lesions from a case of spontaneous blackhead. Eight of these turkeys were 60 days old and four were 68 days old. October 14 (5 days after inoculation). All but one (No. 71) showed well marked swellings on the breast at the site of inoculation. In this single exception an indurated mass situated deep in the muscle was found on the 11th day after inoculation. These turkeys were distributed in four groups of three each, the first group serving as controls, the second receiving neoarsphenamine, the third atoxyl, and the fourth tryparsamide treatment. All three drugs were administered intravenously in three successive doses given at 48 hour intervals, beginning 5 days after inoculation. The neoarsphenamine was dissolved in a small amount of sterile distilled water and injected immediately into the wing vein. A 1 per cent solution of atoxyl and a 25 per cent solution of tryparsamide were prepared by dissolving the required amounts of the salts in sterile distilled water. These solutions were used without further sterilization. The details of the injections together with other data are furnished in Table II.

The dosage employed was based on that previously determined for spontaneous blackhead. Since treatment was commenced in this

experiment before the turkeys showed any impairment of health due to the infection, larger initial doses than those employed might have been more effective. Furthermore, as the turkeys were later on distinctly weaker on account of the extensive lung involvement, the third and last dose was unreasonably large and evidently hastened death in a number of cases.

Neoarsphenamine treatment instituted 5 days after inoculation of blackhead failed to check the development of the local lesion in the breast or to prevent the dissemination of the disease to the lungs. The injection of three doses of 0.04, 0.06, and 0.11 gm. into an 890 gm. turkey was followed by extensive paralysis. The two other neoarsphenamine-treated turkeys also showed pronounced toxic symptoms after the third injection. None recovered.

Atoxyl in the amount administered failed to arrest the development of inoculated blackhead, but possibly larger doses might have been employed. There were no recoveries.

Tryparsamide treatment was attended in one instance by improvement and recovery after two doses, in another by recovery after the injection of sufficient drug to cause partial paralysis, and in the third by general collapse terminating fatally.

There were thus only two recoveries in this series of twelve turkeys inoculated with blackhead, both being turkeys that were treated with tryparsamide.

Late in the experiment, when it was seen that there was no prospect of cure in any except the tryparsamide-treated cases, large doses (2 to 2.5 gm. per kilo of body weight) of this drug were injected into the three controls, and smaller doses (1 gm. per kilo of body weight) into the neoarsphenamine- and atoxyl-treated turkeys. In no instance did the administration of tryparsamide late in the inoculated disease result in a recovery. A period of unfavorable weather undoubtedly hastened the death of the treated turkeys.

The histological study of the lesions from this series indicates that healing of liver lesions may occur under treatment while the disease is still active and extending in the lung and possibly in the local breast lesions. Thus a control turkey (No. 70) which received tryparsamide treatment late in the infection, showed advanced healing of the liver lesions with no demonstrable organisms, whereas in the lungs there

TABLE II.

Group.	Turkey No.	Inoculation.	Weight.	Injections into wing vein.	Result.	Remarks.
1 Controls.	70		Oct. 14. 720 gm. " 18. 780 "		Oct. 29. D.	Inoculated blackhead, involving breast, lungs, and liver.
	71		" 14. 725 " " 18. 780 "		" 24. "	Inoculated blackhead, involving breast and lungs, also cecal and liver lesions due to spontaneous blackhead.
	72		" 14. 780 " " 18. 835 "		" 24. "	Inoculated blackhead, involving breast, lungs, and liver. A core without any microscopic evidence of blackhead in cecum.
2 Treated with neoarsphenamine.	73		Oct. 14. 780 gm. " 16. 805 "	Oct. 14. 0.04 gm. " 16. 0.05 " " 18. 0.08 "	Oct. 21. D.	Inoculated blackhead; lesions of breast, lungs, liver, and pancreas.
	74		" 14. 890 " " 16. 975 "	" 14. 0.04 " " 16. 0.06 " " 18. 0.11 "	" 23. "	Inoculated blackhead; lesions of breast and lungs. Paralysis following last injection suggestive of overdosage.
	75		" 14. 780 " " 16. 835 "	" 14. 0.03 " " 16. 0.04 " " 18. 0.07 "	" 24. "	Inoculated blackhead; lesions of breast and lungs. Spontaneous blackhead with lesions in cecum and liver.
3 Treated with atoxyl.	76	Oct. 9, 1922. Each inoculated in left breast with blackhead liver lesions Oct. 14. A well defined lesion	Oct. 14. 780 gm. " 16. 835 "	Oct. 14. 2 cc. of 1 per cent solution. Oct. 16. 3 cc. of 1 per cent solution. Oct. 18. 6 cc. of 1 per cent solution.	Oct. 25. D.	Inoculated blackhead; lesions of breast and lungs.

3 Treated with atoxyl.	77	developing locally in each, except No. 71, in which the swelling was first noted on Oct. 20.	Oct. 14. 555 gm. " 16. 615 "	Oct. 14. 1.5 cc. of 1 per cent solution. Oct. 16. 2 cc. of 1 per cent solution. Oct. 18. 4 cc. of 1 per cent solution.	Oct. 24. D.	Inoculated blackhead; lesions of breast, lungs, and liver.
	78		" 14. 835 " " 16. 945 "	Oct. 14. 2 cc. of 1 per cent solution. Oct. 16. 4 cc. of 1 per cent solution. Oct. 18. 8 cc. of 1 per cent solution.	" 21. "	Inoculated blackhead; lesions of breast and lungs.
4 Treated with tryparsamide.	79		Oct. 14. 890 gm. " 16. 975 "	Oct. 14. 3 cc. of 25 per cent solution. Oct. 16. 6 cc. of 25 per cent solution.	Recovered.	Breast lesion 2 by 3 by 1 cm.; regressed following second injection. General condition gradually improved although growth somewhat checked.
	80		" 14. 835 " " 16. 865 "	Oct. 14. 4 cc. of 25 per cent solution. Oct. 16. 6 cc. of 25 per cent solution. Oct. 18. 6 cc. of 25 per cent solution.	Oct. 24. D.	Inoculated blackhead; lesions of breast and lungs. Impaired vision and weakness of legs following last injection.
	81		" 14. 610 " " 16. 670 "	Oct. 14. 4 cc. of 25 per cent solution. Oct. 16. 8 cc. of 25 per cent solution. Oct. 18. 9 cc. of 25 per cent solution.	Recovered.	Appetite unimpaired throughout infection. Growth unchecked. Partial paralysis following last injection; mincing gait with hyperextension of toes persists.

were active lesions and moderately numerous apparently healthy organisms. In one of the neoarsphenamine-treated turkeys which received an injection of tryparsamide late in the disease, there were an active breast lesion and extensive disease in the lungs but the liver lesions showed an unusual picture; the affected areas presented a connective tissue reticulum through which extended the dilated sinusoids but in which there was only a trace of necrotic parenchyma. The prompt absorption of the necrotic tissue and the maintenance of circulation in the infected portions are undoubtedly favorable to repair. The histological evidence that blackhead infection is more readily checked in the liver than elsewhere in the body by chemotherapeutic agents indicates the reason that recovery occurs on treatment so much more frequently in the spontaneous than in the inoculated disease.

CONCLUSIONS.

The natural form of blackhead, although very fatal to young turkeys, is favorably influenced by treatment with certain of the newer arsenical compounds.

Neoarsphenamine injected intravenously in toxic doses has a somewhat favorable effect on the course of the spontaneous disease and evidently lowers the mortality. Its instability, its pronounced toxicity for young turkeys, as well as the difficulty of obtaining amounts suitable for the cases to be treated on any one occasion, serve to make its employment impracticable.

Less favorable results were obtained with atoxyl.

Arsenious acid fed daily in small doses failed to prevent blackhead and possibly increased the incidence of infection. In larger doses it failed to cure spontaneous blackhead.

Tryparsamide may be injected in cases of spontaneous blackhead in doses as high as 1 gm. per kilo of body weight either intravenously or subcutaneously without serious toxic effect. Prompt clinical improvement usually follows and the mortality is undoubtedly greatly lowered.

With inoculated blackhead recovery is more difficult to obtain. Birds treated by injections of either neoarsphenamine or atoxyl failed

to recover. There were two recoveries among three tryparsamide-treated cases. An attempt to cure inoculated blackhead at an advanced stage by the injection of tryparsamide failed.

The study of the lesions of treated turkeys shows that tryparsamide, and to some extent neoarsphenamine, have a more pronounced effect on the liver lesions than on the disease in the lung, which accounts for the discrepancy in the results of the treatment of spontaneous and inoculated blackhead. There is histological evidence of prompt absorption of necrotic liver parenchyma; moreover, the protozoa are destroyed more rapidly in the liver than in the lung.

Tryparsamide has the most pronounced curative action on blackhead infection of any of the drugs thus far tested. Either intravenous or subcutaneous injection of appropriate doses of this drug at the first appearance of symptoms should serve to cure the majority of cases of blackhead.

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